ROLE OF NLRP3 IN COLITIS AND COLITIS ASSOCIATED COLORECTAL CANCER

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4. Tanvi Shinde, Ravichandra Vemuri, Madhur D Shastri, **Agampodi Promoda Perera**, Stephen Tristram, Roger Stanley, Rajaraman D Eri, "Probiotic Bacillus coagulans MTCC 5856 spores exhibit excellent in-vitro functional efficacy in simulated gastric survival, mucosal adhesion and immunomodulation", Journal of Functional Foods, January 2019, Volume 52, Pages 100-108. doi.org/10.1016/j.jff.2018.10.031.

5. Ravichandra Vemuri, Rohit Gundamaraju, Tanvi Shinde, **Agampodi Promoda Perera**, Waheedha Basheer, Benjamin Southam, Shakuntla V. Gondalia, Avinash V. Karpe, David J. Beale, Stephen Tristram, Kiran D K Ahuja, Madeleine Ball, Christopher J Martoni, Rajaraman Eri, "Distinct patterns of microbiota, improvements in immunological and short-chain fatty acid profiles with Lactobacillus acidophilus DDS-1 supplementation in aging mice", Nutrients, June 2019, Volume 11, Pages 1297.

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ABBREVIATIONS

AOM	Azoxymethane
APCs	Antigen-presenting cells
ASC	Apoptosis speck protein
BMDM	Bone marrow derived macrophages
CAC	Colitis-associated cancer
CD	Crohn's disease
CHO	Carbohydrates
COX-2	Cyclooxygenase 2
CRC	Colorectal cancer
CRP	C reactive protein
DCs	Dendritic cells
DDR	DNA damage response
DEX	Dexamethasone
DSS	Dextran sodium sulphate
DSB	Double-strand breaks
IECs	Intestinal epithelial cells
ENU	N-ethyl-N-nitrosourea
ER	Endoplasmic reticulum
ENS	Enteric nervous system
H&E	Haematoxylin and eosin
IBD	Inflammatory bowel diseases
IFN-γ	Interferon gamma
LPMC	Lamina propria macrophage cells
LPS	Lipopolysaccharide
mRNA	Messenger ribonucleic acid
Muc2	Mucin 2 gene
MLNs	Mesenteric lymph nodes
MIP-1α	Macrophage inflammatory protein – 1 alpha
NF-κB	Nuclear factor-κB
NLRP3	NOD-like receptor protein 3
NOD	Nucleotide-binding oligomerization domain
Nrf2	Nuclear factor erythroid 2-related factor 2
NQO1	Quinoneoxido reductase 1
PAMP	Pathogen-associated molecular pattern
PI3K	Phosphatidylinositol 3-kinase
PPARγ	Peroxisome proliferator activated receptor-γ
PRRs	Pattern recognition receptors
qPCR	Quantitative polymerase chain reaction
RONS	Reactive oxygen and nitrogen species
SCFA	Short chain fatty acid
SPF	Specific pathogen-free
STAT3	Signal transducer and activator of transcription 3
TCR	T-cell receptor
TGF-β1	Transforming growth factor beta1
TME	Tumor micro environment
TNBS	2, 4, 6-trinitrobenzene sulfonic acid
TNF-α	Tumour necrosis factor alpha
UC	Ulcerative colitis
UPR	Unfolded-protein response
VEGF	Vascular endothelial growth factor
WT	Wild Type

GENERAL ABSTRACT

Inflammatory bowel disease (IBD) is an idiopathic group of chronic disorders characterised by inflammation of the small intestine and colon. In Australia, more than 85,000 people live with IBD and by the year 2022 it is expected that this number will have surpassed 100,000. The two major types of IBD are Crohn's disease (CD) and ulcerative colitis (UC). Both diseases are characterised by a series of relapses and remissions where the inflammation in the gastrointestinal tract becomes so severe that patients need hospitalisation or even surgery.

A major complication of chronic inflammation (colitis), either by recurrent CD or UC is the development of colitis-associated colorectal cancer (CAC). Crohn and Rosenberg first reported the ulcerative colitis case associated with colorectal cancer development in 1925, thereafter many studies have been published linking gut inflammation as an important predisposing factor for the development of colorectal cancer. However, the understanding of specific underlying mechanism of how chronic inflammation is connected to the initiation and progression of colon cancer is yet to be established.

Although the exact aetiology of IBD has yet to be elucidated, a defective innate immune system has been proposed as a primary mechanism in colitis. As an important arm of the innate immune system inflammasomes particularly the well characterised NLRP3, is involved in gut homeostasis and inflammatory pathologies. However, the role of NLRP3 in these processes is not well understood.

Investigations on the role of NLRP3 in colitis is still controversial and inconclusive with some studies showing a protective role while other studies demonstrate a detrimental effect of NLRP3 activation. The reason for the discrepancies observed in the *Nlrp3*$^{-/-}$ chemically induced colitis mouse model phenotype could be due to differences in length and concentration of chemical treatment or baseline differences in the composition of the intestinal microbiota in experimental mice. Thus, the overall aim of the PhD project was to address the above issues and define the role of NLRP3 in the development of colitis.

This doctoral research thesis consists of seven chapters. Chapter 1 is an overall introduction to the rationale of the research, hypothesis and aims of the research project. Chapter 2 is a review article that critique the literature and summarises the role of NLRP3 in intestinal

inflammation and CAC. Chapter 2a is a detailed description of the Winnie mouse model and methodology of generation and genetic confirmation of the novel mouse model Winnie x *Nlrp3*$^{-/-}$.

Chapter 3 is a review article on background of current experimental NLRP3 inhibitors that have been investigated in IBD experimental models and the potential mechanism of action of these inhibitors. Chapter 4 is a research article that describes the experiments and data generated that detail the effect of MCC950, a specific small molecule inhibitor of NLRP3 inflammasome on colonic inflammation in spontaneous colitis Winnie mice.

Chapter 5 details research experiments and data generated that characterise clinical parameters, histopathology, biochemical and cytokine profile of the novel mouse model Winnie x *Nlrp3*$^{-/-}$ mouse model. In chapter 6, I further explored the molecular mechanisms that lead to CAC by investigating the hyperactive molecular pathways by means of analysing the protein and gene expression, metabolomics and microbiota changes in the CAC mouse model Winnie x *Nlrp3*$^{-/-}$. Finally, chapter 7 is a comprehensive discussion of all the results obtained from the research objectives and includes potential directions for future work arising from this research thesis.

Current treatment regimen for IBD utilise anti-inflammatory drugs, immune system suppressors and antibiotics or a combination of these. However, these therapeutics lead to several adverse effects, remission or significant non-responsiveness leading to colectomy which is an emergency surgery with a high rate of morbidity. Thus, there is an urgent need to develop potent drugs with novel mechanisms of action. Given the evidence that aberrant NLRP3 activation is involved in the progression of IBD, targeting the activation pathway is a promising strategy for the development of novel effective therapeutics for IBD. Therefore, I reviewed past literature and discussed all the experimental NLRP3 inhibitors that has been investigated in IBD experimental animal models. The most salient finding of the review article was that all experiments were conducted in chemically induced colitis models with inhibitors that were nonspecific to the NLRP3 inflammasome and therefore was unable to specifically define its role towards IBD.

Therefore, I investigated the therapeutic effect of a NLRP3 inhibitor, MCC950 in a spontaneous chronic colitis mouse model Winnie. Extensive studies in Winnie have proven it to be an appropriate murine model to study IBD and its pathogenesis. MCC950 is a potent, highly specific small molecule inhibitor of both canonical and noncanonical

activation of NLRP3 inflammasome and has been evaluated in a multitude of NLRP3 driven inflammatory diseases. However, the effect of MCC950 on colitis has not yet been reported. To my knowledge this is the first time a specific NLRP3 inhibitor has been applied to colitis and I was able to determine the contribution of anti-inflammatory effects resulting exclusively from inhibition of canonical and non-canonical NLRP3 inflammasome activation in colitis. Mice were orally administered with 40 mg/kg of MCC950 for three weeks at chronic stage of colitis. The treatment significantly ameliorated colitis with improved body weight gain, colon length, ratio of colon weight to body weight, and disease activity index. Histopathological scores of MCC950 treated Winnie mice were significantly reduced suggesting not only attenuation of ongoing colitis but also delay of disease onset. MCC950 significantly suppressed IL-1β and IL-18 cytokine expression at both mRNA and protein levels in Winnie colons. Additionally, MCC950 also effectively suppressed the release of proinflammatory cytokines (IL1-α, IL17, TNF-α and IFN-γ) and chemokine (MIP1a) in mucosal explants. Moreover, MCC950 treatment resulted in a significant decrease of IL-1β release and activation of caspase-1 in Winnie explants and *in vitro* macrophage cells isolated from these mice. Taken together, the results illustrate the efficacy of MCC950 in the treatment of murine ulcerative colitis and provides a potential avenue for a novel therapeutic agent for human inflammatory bowel diseases.

With the successful discovery of the therapeutic potential of the specific NLRP3 inhibitor MCC950, I hypothesised that the absence of NLRP3 inflammasome in the spontaneous colitis mouse model Winnie would ameliorate colitis. To conduct this work, I generated a novel mouse model by knocking out the *Nlrp3* gene in Winnie, with a defined microbiota, to elucidate the functional role of NLRP3 inflammasome in colitis.

Interestingly, detailed phenotypical analysis of Winnie x *Nlrp3*$^{-/-}$ colon at 12 and 16 weeks showed spontaneous multiple colonic tumours. Winnie x *Nlrp3*$^{-/-}$ mice had significantly shorter colons, and a higher ratio of colon weight to length and colon weight to body weight compared to control groups indicating the severity of colitis and tumorigenesis. Histopathology of Winnie x *Nlrp3*$^{-/-}$ colon revealed severe crypt distortion and goblet cell depletion with high-grade dysplasia and invasive carcinoma regions. Analyses of colonic tissue homogenates by biochemical assays showed increased activity of myeloperoxidase, Nitric Oxide and serum C-reactive protein consistent with human CAC. RNA was extracted from colonic tissue segments converted to cDNA and analysed for proinflammatory and

cancer biomarker gene expression using PCR microarray. Upregulated biomarkers *Bcl2, Sod2, Pparγ, Myc*, *Birc5* and *Cdk2* were confirmed by individual qPCR. Colonic organ cultures were performed, and the supernatants were assayed via Bio-Plex and results identified differential expression of proinflammatory cytokines, chemokines and cancer biomarkers. Detailed immunohistochemistry revealed high-grade dysplasia and adenocarcinoma regions with increased expression of DNA damage biomarkers anti-Oxoguanine 8, anti-gamma H2A.x and oxidative stress biomarkers anti-NQ01, anti3-Nitrotyrosine. Immunofluorescence for Ki-67, VEGF and Survivin biomarkers showed an increased expression indicating cell proliferation, angiogenesis, and anti-apoptotic activity respectively, validating Winnie x *Nlrp3*$^{-/-}$ as a CAC model. Protein analysis of colonic tumours and Western blot results showed upregulation of Wnt/β-catenin and PI3K/ AKT pathways as the potential molecular mechanism of CAC. Faecal microbiota analysis revealed significant increase in colitogenic members such as *Akkermensia muciniphila* in the phylogenetic architecture in Winnie x *Nlrp3*$^{-/-}$ mice while metabolomics profiling revealed upregulation of key metabolites and significant decrease of beneficial short chain fatty acids. These results provided confirmation that NLRP3 is a negative regulator of tumorigenesis during CAC.

In summary, the study has generated new data and knowledge that defines the NLRP3 inflammasome as a double-edged sword in colitis and CAC. Specific chemical inhibition of an over active NLRP3 inflammasome in chronic colitis attenuated severity of the disease whereas genetic ablation of NLRP3 gene in the same colitis model lead to CAC. This highlights the critical function of NLRP3 inflammasome as an innate immunity guardian in the maintenance of gut homeostasis. Finally, the results stress the importance of evaluating the pharmacokinetics and long-term effect of novel NLRP3 inhibitors designed for chronic inflammatory diseases in clinically relevant experimental models before progressing to human clinical trials.

Chapter 1

Introduction

1.1 NLRP3 inflammasome in Intestinal Inflammatory Diseases

Inflammatory bowel disease (IBD) is a group of life long gastrointestinal disorders characterised by inflammation of the small intestine and colon. IBD is a global disease, with over 1 million patients in the USA and 2.5 million in Europe (Kaplan, 2015). IBD is an emerging disease in newly industrialized countries in Asia, South America and Middle East with rising incidence in every continent, with Australia having one of the highest prevalence with more than 85,000 IBD patients and by the year 2022 it is expected that this number will have surpassed 100,000 (Wilson et al., 2010, Loftus, 2004). In 2013, the annual economic and financial cost associated with managing IBD in Australia was estimated at 3.1 billion (Australia, 2013).

The two major types of IBD are Crohn's disease (CD) and ulcerative colitis (UC). Both diseases are characterised by a series of relapses and remissions. CD is a transmural, granulomatous condition commonly involving the ileum and the colon. In contrast, UC specifically involves the colon and rectum of the intestine and manifests as superficial inflammation confined to the mucosal and submucosal layers of the intestinal wall (Blumberg and Strober, 2001, Monteleone and Neurath, 2012). Overlapping symptoms of CD and UC are chronic relapsing flares associated with rectal bleeding, abdominal pain and diarrhoea (Monteleone and Neurath, 2012 , Strober, 2013).

A major complication of chronic inflammation, either by recurrent CD or UC is the development of colitis-associated colorectal cancer (CAC) (Triantafillidis et al., 2009, Parian and Lazarev, 2015). Crohn and Rosenberg first reported the ulcerative colitis case associated with colorectal cancer development in 1925 (Crohn and Rosenberg, 1925b), thereafter studies have shown gut inflammation as an important predisposing factor for the development of colorectal cancer (Karin and Greten, 2005, Dupaul-Chicoine et al., 2010). The cumulative risk of ulcerative colitis associated cancer is estimated to be at 1.6 % at 10 years, 8.3 % at 20 years and 18.4 % at 30 years (Eaden et al., 2001). However, the precise mechanism by which chronic colitis develop in to colorectal cancer is yet to be established.

Although the exact aetiology of colitis has yet to be elucidated, recent studies identify a defective innate immunity as the primary mechanism in chronic mucosal inflammation in IBD (Xavier and Podolsky, 2007, Xu et al., 2014, Marks and Segal, 2008). The innate immune response to cell stress or infection depends on the activation of receptors called pattern recognition receptors (PRRs) (Halle et al., 2008). The two main types of receptors are, Toll-like receptors (TLRs) and Nod-like receptors (NLRs) (Schroder and Tschopp, 2010, Martinon et al., 2009). Activation of these receptors initiate an immune response which involves activation of the inflammasome complex and a cascade of pro-inflammatory cytokines and an adaptive immune response (Ranson and Eri, 2013).

Among the NLR activated inflammasomes, NLRP3 is the best characterized and is closely associated with inflammatory diseases (Duewell et al., 2010, Rajamaki et al., 2010). The NLRP3 inflammasome is a multimolecular platform constituting of NLRP3 protein encoded by the NLRP3 gene and an adaptor protein ASC (known as the apoptosis-associated speck-like protein containing a CARD) and procaspase-1. Activation of the NLRP3 inflammasome leads to activation of caspase-1 and the consequent cleavage and secretion of IL-1β and IL-18 (Martinon et al., 2002) proinflammatory cytokines (Figure 1.1).

These proinflammatory cytokines are associated with increased colitis (Villani et al., 2009, Schoultz et al., 2009) and colorectal cancer (Allen et al., 2010) development. Genetic association studies show that polymorphisms in the IL-1β gene cluster significantly increases the risk of developing a variety of cancers, including gastric cancer (Barber et al., 2000, El-Omar et al., 2001). IL-1β levels are significantly altered in patients suffering from either acute or chronic gastrointestinal inflammation and have been implicated in tumour angiogenesis, progression, and metastasis (Bioque et al., 1995, Casini-Raggi et al., 1995). Studies on IL-18 polymorphisms have confirmed its association with the increased susceptibility to CD (Tamura et al., 2002). In addition, in sites of active intestinal inflammation in CD, IL-18 (Monteleone et al., 1999) and IL-1β (Papadakis and Targan, 2000) has been shown to be overexpressed.

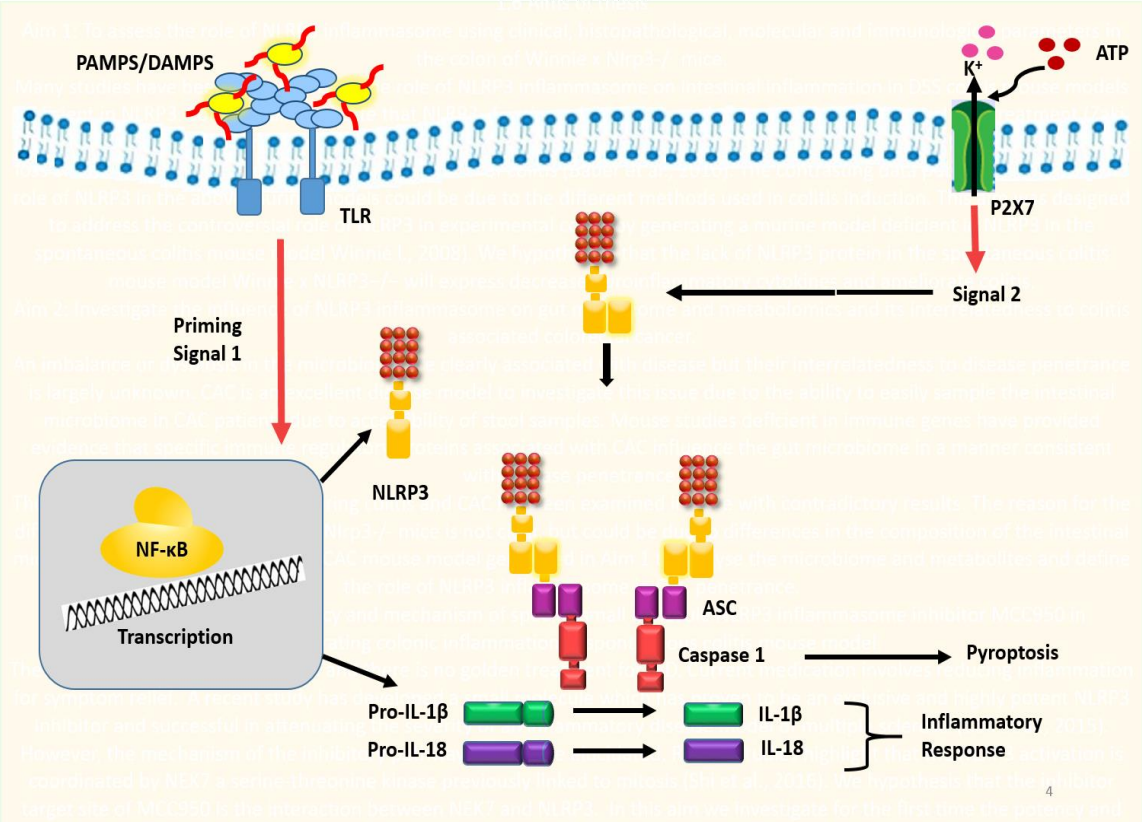

Figure 1.1: Schematic illustration of the Canonical activation pathway of NLRP3 inflammasome.

Many studies have assessed the role of NLRP3 inflammasome on intestinal inflammation in Dextran Sodium Sulphate (DSS) colitis mouse models deficient in NLRP3. Some studies indicate that *Nlrp3*$^{-/-}$ mice exhibited severe inflammation following oral DSS treatment (Zaki et al., 2010a, Dupaul-Chicoine et al., 2010, Allen et al., 2010, Hirota et al., 2011). Controversially, competing studies have reported that the loss of NLRP3 was protective in the DSS mouse model of colitis (Bauer et al., 2010, Elinav et al., 2011). Therefore, there is a crucial need to resolve the role of NLRP3 in colitis.

The contrasting data published regarding the role of NLRP3 in the above experimental models could be due to the different chemical methods used in colitis induction and alteration in the gut microbiota between different animal facilities. Thus, in the current work, I characterize and explore the role of NLRP3 mediation in a spontaneous colitis mouse model with a defined microbiota.

1.2 Summary

Studies in Winnie investigating IBD pathogenic mechanisms have proven that Winnie spontaneous colitis closely resemble human ulcerative colitis, making these mice a valuable tool in IBD research and treatment. Thus, we used this model to investigate the therapeutic potential of MCC950, a specific NLRP3 inhibitor in ameliorating colitis.

The Winnie mouse model is remarkable in that colitis results from an intestinal epithelial defect whilst the underlying immune system is normal and does not display major disturbances to immunity as seen in many other mouse models of IBD. Therefore, it will be easier to interpret pathological changes specifically resulting from genetic deletion of *Nlrp3* and hence was chosen to be used in developing the Winnie x *Nlrp3*$^{-/-}$ model for the research study.

The project will give a comprehensive understanding of the role of NLRP3 inflammasome in colonic inflammation in IBD. This has potential implication not only in IBD pathogenesis and treatment but also in the understanding the role of NLRP3 inflammasome in colitis associated colorectal cancer.

1.3 Hypothesis

Inactivation of the NLRP3 inflammasome will ameliorate colitis in the spontaneous colitis mouse model.

1.4 Aims

Aim 1: To investigate the potency and mechanism of specific small molecule NLRP3 inflammasome inhibitor, MCC950 in ameliorating colonic inflammation in spontaneous colitis mouse model.

The exact cause of IBD is not known and there is no golden treatment for IBD. Current experimental NLRP3 inhibitors have shown to decrease symptoms of colitis in mouse models however they are not exclusive inhibitor of NLRP3 inflammasome. A recently developed small molecule is proving to be an exclusive and highly potent NLRP3 inhibitor and has shown success in attenuating the severity of an inflammatory disease model of multiple sclerosis (Coll et al., 2015). However, the mechanism of the inhibitory pathway

is yet to be elucidated. In this aim, I investigate for the first time the potency and specific NLRP3 inhibitory action of MCC950 to ameliorate colitis and investigate the mechanistic pathway.

Aim 2: To assess the role of NLRP3 inflammasome in colitis by analysing clinical, histopathological, molecular and immunological parameters of Winnie x *Nlrp3*$^{-/-}$ colon.

This aim was designed to address the controversial role of NLRP3 in experimental colitis by investigating the phenotype of a novel murine model deficient in NLRP3 in the spontaneous colitis mouse model Winnie. To my knowledge this is the first study of genetic ablation of *Nlrp3* in a spontaneous colitis mouse model resembling human UC and warrants extensive investigation.

Aim 3: To investigate the influence of NLRP3 inflammasome on gut microbiome and metabolomics and its interrelatedness to colitis associated colorectal cancer in mouse model.

An imbalance or dysbiosis in the microbiome is associated with spontaneous colitis and increased susceptibility to CAC. Mouse studies deficient in immune genes have provided evidence that specific immune regulatory proteins associated with CAC influence the gut microbiome in a manner consistent with disease penetrance.

The role NLRP3 plays in dysbiosis during colitis and CAC has been examined in *Nlrp3*$^{-/-}$ mice with contradictory results. The reason for discrepant results is not clear but could be due to differences in the composition of the intestinal microbiome. Here, we used the CAC mouse model generated in Aim 2 to analyse the microbiota and metabolites and define the role of NLRP3 inflammasome in CAC penetrance.

Chapter 2

Literature Review

NLRP3 inflammasome in colitis and colitis-associated colorectal cancer

2.1 Abstract

A low level of inflammation is an integral part of the balance between the immune system and the microbiota in the high antigen environment of the gastrointestinal tract and maintains homeostasis. A failure of this balance can lead to chronic intestinal inflammation and increase the chances to develop colorectal cancer significantly. The underlying mechanisms that link inflammation and carcinogenesis are not clear but the molecular platforms of the inflammasomes have been implicated. Inflammasomes are molecule complexes that are assembled in response to microbial components or cellular danger signals and facilitate the production of bioactive pro-inflammatory cytokines. One inflammasome, NLRP3, has been analysed extensively in its contribution to colitis and has been shown to be associated with the development of colitis associated colorectal cancer. This review will summarise the role of NLRP3 in intestinal inflammation, discuss some of the triggers of inflammation in the gastrointestinal tract such as diet and introduce some opportunities to use this inflammasome as therapeutic target for the treatment of colitis and colitis-associated colorectal cancer.

2.2 Introduction

Colorectal cancer (CRC) is the third most common malignancy worldwide (Ferlay et al., 2015) and presents with a high mortality rate (Siegel et al., 2016) due to rapid cancer progression with late diagnosis at an advanced tumour stage (Siegel et al., 2014). An inflammation-associated form of CRC, colitis-associated CAC has been recognised as a complication of inflammatory bowel disease (IBD) (Parian and Lazarev, 2015, Triantafillidis et al., 2009). These chronic inflammatory, idiopathic disorders are characterised by significant inflammation of small intestine and colon and are becoming more prevalent and more severe due to global adoption of western diet, the increasing use of nonsteroidal anti-inflammatory medications and an ageing population (Taleban et al., 2015). Furthermore, the increasing incidence and prevalence of IBD in children makes it

an important paediatric chronic disease (Nasiri et al., 2017) with an increased risk of developing CAC (Peneau et al., 2013).

The two primary types of IBD are Crohn's disease (CD) and ulcerative colitis (UC). CD commonly affects the small intestine or colon, however it can affect all parts of the gastrointestinal tract from the oesophagus to the anus involving all layers of the intestinal wall. Complications characteristic for CD are strictures, fistulae and fissures. UC is characterised with inflammation that is limited to the mucosa of the colon and presents with bleeding ulcers and that can result in the perforation of the colon (Cosnes et al., 2011, Mulder et al., 2014).

2.3 Innate immunity, Inflammatory Bowel Disease and Cancer

Crohn and Rosenberg first reported an ulcerative colitis case associated with colorectal cancer development in 1925 (Crohn and Rosenberg, 1925a). Subsequently, many studies have been published indicating gut inflammation as an important factor predisposing the development of colorectal cancer (Dupaul-Chicoine et al., 2010, Karin and Greten, 2005). The cumulative risk of UC-associated cancer is estimated to be at 1.6% at 10 years, 8.3% at 20 years and 18.4% at 30 years (Eaden et al., 2001) correlating CAC directly to the extent and duration of colitis. Nevertheless, the exact mechanism of how chronic inflammation is connected to the development of colitis-associated colorectal cancer (CAC) has yet to be established.

Chronic inflammation in IBD progresses to CAC with constant overproduction of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α (Becker et al., 2004, Popivanova et al., 2008), chemokines and DNA damaging reactive oxygen and nitrogen species. These inflammatory effector molecules promote aberrant intestinal epithelial cell proliferation, survival and angiogenesis and lead to epithelial dysplasia and ultimately to a formation of invasive tumours (Grivennikov, 2013). Therefore, understanding and modulating the mechanisms of chronic mucosal inflammation will be the key to preventing the progression to CAC (Foersch and Neurath, 2014).

Although the exact aetiology of chronic intestinal inflammation is not yet known, recent studies support the hypothesis of a defective innate immune response as the primary mechanism in chronic mucosal inflammation (Asquith and Powrie, 2010). The innate immune response controls the intestinal microbiota and provides initial resistance to

invading pathogens whilst maintaining homeostasis (Ignacio et al., 2016). Beyond anatomical barriers such as the skin and mucosa, various cellular components of the innate immune system such as epithelial cells, macrophages, dendritic cells (DC) and neutrophils are in the intestinal wall and intestinal lymphoid organs including Peyer's Patches and mesenteric lymph nodes. These cell populations keep the microbial occupants of the intestines under surveillance with the help of extracellular and cytosolic pattern recognition receptors (PRRs).

The PRRs comprise the pro-inflammatory membrane-bound Toll-like receptors (TLR) and the cytosolic sensory protein complexes consisting of NOD-like receptors (NLR), the RNA sensing retinoic acid-inducible gene-1 receptors (RLR) and the pyrin and HIN domain (PYHIN) receptor family which includes the AIM2-like receptor (ALR) and C-type lectins (Ranson et al., 2017). These receptors recognise pathogen-associated molecular patterns (PAMPs) or host derived danger-associated molecular patterns (DAMPs). Receptor engagement causes cellular activation of various effector mechanisms ranging from microbicidal molecules and phagocytosis to activation of large multiprotein complexes called inflammasomes.

2.4 Biology of inflammasomes

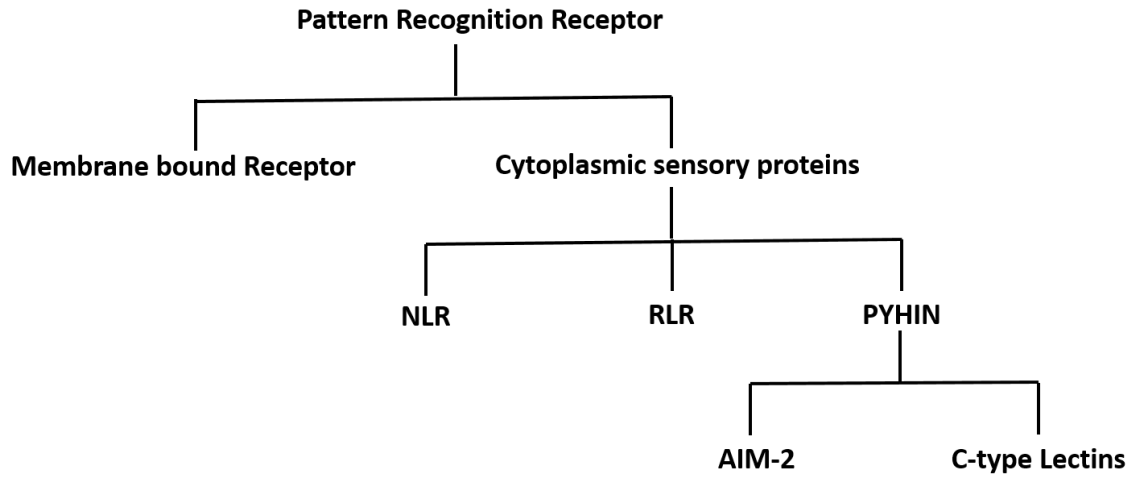

Figure 2.1: PRRs that initiate innate immunity.

Inflammasomes have emerged as a central feature in innate immunity (Martinon et al., 2002) and are involved in pathogen clearance, maintain tissue homeostasis and stimulate an adaptive immune response that removes tumour cells. These large cytosolic protein complexes can be divided in the NLR, the PYHIN or the ALR family (Figure.2.1) and comprise inflammatory caspases which undergo autocatalytic activation and initiate inflammatory signalling cascades that activate protease caspase-1 and releases pro-inflammatory cytokines IL-1β and IL-18 (Latz et al., 2013, Ranson and Eri, 2013).

This sequence of activating events is culminating in the initiation of pyroptosis, a Gasdermin-D-mediated form of inflammatory programmed cell death (Man et al., 2017). The functionally most completely characterised family member of the NLR is the NOD-like receptor family, pyrin domain containing protein 3 (NLRP3) (Table 2.1).

Table 2.1: NLR subfamilies and their functions

Family	Subtypes	Role in immunity	References
NOD	6 subtypes	Defence against bacterial and viral infection. NLRC3 is associated with Colitis, CAC and CRC. NLRC4 has been correlated with melanoma	(Janowski et al., 2013, Karki et al., 2016, Kobayashi et al., 2005, Loving et al., 2009, Viala et al., 2004, Zhang et al., 2014b)
IPAF	2 subtypes	NAIP–NLRC4 together prevent against bacterial infection	(Janowski et al., 2013, Zhao and Shao, 2015)
NLRP	14 subtypes	Associated with defence against various bacterial and viral infection. NLRP3, 6 and 12 have been associated with colitis and CAC. NLRP3 is associated with various manifestations of metabolic syndrome	(Allen et al., 2010, Anand et al., 2012, Chavarria-Smith and Vance, 2015, Janowski et al., 2013, Vladimer et al., 2012)

The association of inflammasomes with intestinal inflammation and CAC has been demonstrated with expression analysis of human colon cancer samples which shows lower expression levels for NLRP and AIM2 family members (Liu et al., 2015, Ranson and Eri, 2013). Genetic ablation of these inflammasomes in the DSS/AOM CAC murine model suggested that they act to suppress intestinal inflammation associated tumorigenesis essentially through inhibiting cellular proliferation and driving cell death (Allen et al.,

2010, Chen and Nunez, 2011, Karki et al., 2017, Normand et al., 2011, Wilson et al., 2015, Zaki et al., 2011c). Mice deficient in inflammasome components such as apoptosis associated speck-like protein containing a caspase activation and recruitment domain (ASC), caspase-1 and caspase-11, IL-18 or IL-18r, exhibit increased colitis and tumorigenesis compared to wild-type (WT) mice in the azoxymethane dextran sulphate sodium (AOM-DSS) model (Dupaul-Chicoine et al., 2010, Salcedo et al., 2010, Williams et al., 2015, Zaki et al., 2010b). These studies highlight the importance of inflammasome pathways in the modulation of colitis and the suppression of CAC.

2.5 Mechanism of action: NLRP3 in the innate immune response

Polymorphisms of the *Nlrp3* gene are associated with poor survival in CAC patients and the specific role of NLRP3 in the processes leading to tumorigenesis is not well understood (Ungerbäck et al., 2012). Therefore, we will discuss the current literature on the specific contribution of NLRP3 to the modulation of the intestinal microbiota and intestinal pathologies of colitis and CAC.

The NLRP3 inflammasome is a multiprotein platform comprising the NLRP3 protein, the adaptor protein ASC and pro-caspase-1. The NLRP3 protein itself contains a nucleotide-binding and oligomerization domain (NBD or N ACHT), carboxy-terminal leucine-rich repeat (LRR) at the C-terminus and a PYD (Leemans et al., 2011). The inflammasome assembly is inhibited by the LRR domain. This activity is disabled by the activating signal either from PAMPS or DAMPS, whereas the NBD is required for homo or hetero-oligomerization that leads to the activation of caspase-1 from the inactive zymogen pro-caspase-1 and the subsequent auto-cleavage and secretion of the pro-inflammatory cytokines IL-1β and IL-18 (Martinon et al., 2002, Schroder and Tschopp, 2010).

Triggers for activation of NLRP3 inflammasome include a diversified array of unrelated molecular structures (PAMPs) such as microbial cell wall components including lipopolysaccharide (LPS) and muramyl dipeptide (MDP), nucleic acids, pore-forming toxins, DAMPs, ATP and crystalline substances such as uric acid, oxidised mitochondrial DNA. As the NLRP3 inflammasome assembles in response to these molecules, it has been proposed that it responds to a common cellular distress signal, instead of a direct interaction with cognate ligands (Halle et al., 2008, Mariathasan et al., 2006, Martinon et al., 2006, Shimada et al., 2012) (Figure 2.2).

Three key mechanistic pathways have been suggested for triggering the activation of NLRP3 inflammasome. Firstly, pore formation and potassium efflux (Petrilli et al., 2007), secondly, lysosomal destabilisation (Okada et al., 2014) and thirdly, mitochondrial reactive oxygen species (mROS) generation (Gurung et al., 2015). Importantly, the presence of ROS has been implicated in the activation of the NLRP3 inflammasome and has been linked to cancer promotion (Fang et al., 2009).

Canonical activation of NLRP3 occurs in two steps via both transcriptional and post-transcriptional processes. The first signal (Signal 1) is provided predominantly in a PAMPs-dependent manner. A frequently used model is the lipopolysaccharide (LPS)-induced activation of the TLR4/NF-κB pathway. This step is termed "priming" and causes a transcriptional up-regulation of the transcription of *Nlrp3* mRNA and for *pro-il-1β* and *-il-18* (Latz et al., 2013) (Figure 2.2).

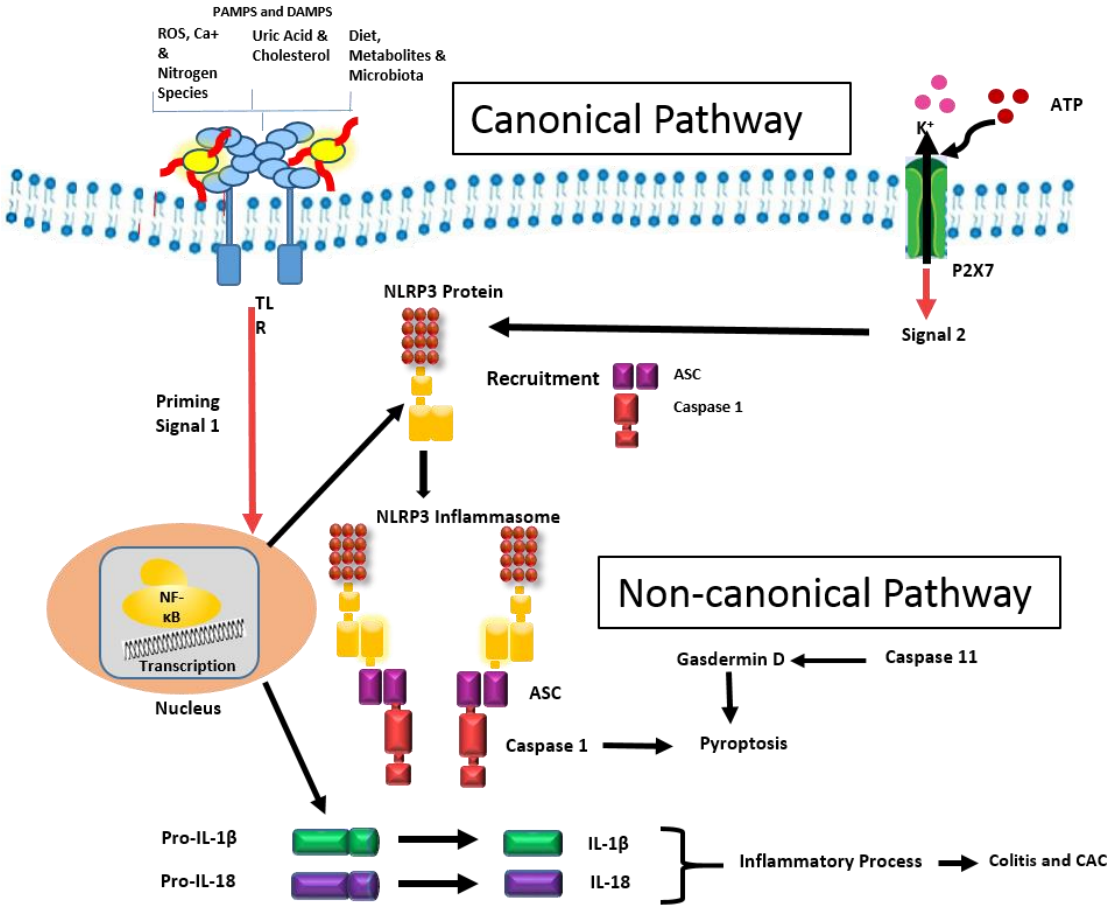

Figure 2.2: Canonical and non-canonical activation of NLRP3.

In a second step, intracellular sensing of specific ligands (DAMPs) leads to the recruitment and oligomerization of the key adaptor protein, ASC, which, through its caspase activation and recruitment domain (CARD) facilitates the subsequent recruitment and activation of caspase-1. In a final activating step this protease catalyses the proteolytic cleavage of inactive pro-IL-1β or pro-IL-18 proteins into secreted bioactive cytokines which initiate a plethora of potent inflammatory responses. Furthermore, activation of caspase-11 induces Gasdermin-D-mediated pyroptosis, a form of cell death frequently observed during invasion by gram-negative and gram-positive pathogens (Figure 2.2).

In addition to canonical inflammasomes comprising NLRP3, ASC and caspase-1, recent studies have identified an alternative non-canonical NLRP3 inflammasome which consists of caspase-11 (caspase-4 and caspase-5 in human) and not caspase-1. The non-canonical pathway is seen in infections with gram-negative bacteria, where caspase-11 binds directly to cytosolic LPS. This promotes inflammasome-independent pyroptosis and the assembly of the NLRP3 inflammasome and activation of caspase-1 to cleave pro-IL-1β and pro-IL-18 into secreted bioactive cytokines (Kayagaki et al., 2011, Rathinam et al., 2012b) (Fig. 2).

IL-1β and IL-18 are important pro-inflammatory mediators of the mucosal inflammatory response. The presence of IL-1β can induce various cellular activities, including the proliferation, differentiation and apoptosis of both immune and non-immune cells (Huber et al., 2012, Vela et al., 2002). Additionally, IL-1β can co-stimulate IL-6 production which acts as a growth factor for B cell proliferation and initiate the release of other pro-inflammatory cytokines such as TNF-α, IL-23 and IL-6 that can polarise the adaptive immune response to a Th2 or a Th17 response depending on the antigenic environment (Dinarello, 2009).

The importance of IL-1β in the pathogenesis of colitis has been well established (Ning et al., 2015). In addition, numerous studies have revealed that secretion of IL-1β is elevated in the sera of patients with IBD (Sartor, 1994) and mice subjected to DSS-induced colitis (Bauer et al., 2010). Importantly inhibition of IL-1β has shown to alleviate DSS induced colitis (Siegmund et al., 2001b).

Other studies have indicated that in contrast to IL-1β, IL-18 plays a major role in suppressing colitis and CAC. Polymorphisms of the *il-18* gene have confirmed a strong association of this cytokine with an increased susceptibility to CD (Tamura et al., 2002).

This suggests that IL-18 signalling provides protection against a development of intestinal inflammation. Mice deficient for IL-18 and IL-18 receptor were hyper-susceptible to DSS-induced colitis, which was associated with higher mortality rates and more severe histopathological changes (Takagi et al., 2003). In a similar study, *Il-18*$^{-/-}$ and *Il-18r*$^{-/-}$ mice also developed severe DSS-induced colitis with high lethality and more histopathological abnormalities and were more susceptible to AOM/DSS-induced colon tumorigenesis as compared to WT mice, suggesting an essential and protective role of IL-18 signalling in colorectal cancer (Salcedo et al., 2010). MyD88 KO mice, which are defective in both IL-1β and IL-18 production and the downstream signalling of their respective receptors, showed increased colonic epithelial proliferation and colorectal tumorigenesis (Salcedo et al., 2010).

The explanation proposed was that the increase in tumorigenesis was caused by a decrease in DNA damage response genes causing insufficient response to DNA damage. Additionally, IL-18 is a key mediator in epithelial regeneration by the upregulation of adhesion molecules (Stuyt et al., 2003) during the early stages of colitis (Allen et al., 2010, Hirota et al., 2011, Nowarski et al., 2015, Zaki et al., 2010a). Besides its indirect tumour-suppressive role in CAC, IL-18 has been associated with a T helper 1-skewed immune-stimulatory, anti-tumorigenic response through its ability to induce IFN-γ (Okamura et al., 1995) and its effects on enhancing the cytosolic activity of cytotoxic T cells and NK cell response (Chaix et al., 2008, Novick et al., 2013, Takeda et al., 1998). Furthermore, high levels of IL-18 have been detected in lamina propria mononuclear cells (LPMCs) and colon epithelial cells of patients with Crohn's disease (Monteleone et al., 1999, Pizarro et al., 1999).

In an experimental T cell-mediated colitis model administration of a recombinant IL-18 antisense-expressing adenoviruses was able to reduce IL-18 and suppress IFN-γ thus ameliorating colitis *in vivo* (Wirtz et al., 2002). The connection between IL-18 and IFN-γ was supported by an analysis of LPMCs from *Il-18*$^{-/-}$ mice which featured an exacerbated form of DSS colitis and produced a significantly higher amount of IFN-γ (Takagi et al., 2003). Furthermore, the in murine colitis models neutralisation of IL-18 has shown to ameliorate intestinal inflammation (Siegmund et al., 2001a, Sivakumar et al., 2002) and may therefore an interesting candidate for a targeted immunotherapy of human intestinal inflammatory diseases. These experiments have highlighted the dual role of IL-18 in

intestinal homeostasis and colitis. Early in the mucosal immune response, its expression by IECs and LPMCs indicates a protective, local role through epithelial regeneration and proliferation in response to injury. In chronic inflammation its excessive production can enhance inflammation, which potentially promotes tumorigenesis and tumour growth (Reuter and Pizarro, 2004).

2.6 The role of NLRP3 in colitis and colitis-associated cancer: lessons from mouse models

NLRP3 inflammasome and its activation in intestinal pathologies have been investigated predominantly using mouse models of chemically induced intestinal inflammation and CAC specifically the 2,4,6-trinitrobenzene sulphonic acid (TNBS) and the dextran sulphate sodium (DSS) models of inflammation, and the azoxymethane (AOM or AOM/DSS) model of CAC induction respectively (Table 2.2).

Table 2.2: Examples of colitis and CAC mouse models

Mouse Model	Trigger	Role of NLRP3	Reference
Nlrp3$^{-/-}$	DSS/TNBS	Protective	(Zaki et al., 2010a)
Nlrp3$^{-/-}$	DSS	Protective	(Allen et al., 2010)
Nlrp3$^{-/-}$	DSS	Protective	(Hirota et al., 2011)
Nlrp3$^{-/-}$	DSS	Harmful	(Bauer et al., 2010)
Nlrp3$^{-/-}$	DSS	Harmful	(Elinav et al., 2011)
Nlrp3$^{-/-}$	Oxazolone	Protective	(Itani et al., 2016)
Nlrp3^{R258W}x *Rag1*$^{-/-}$	Spontaneous	Protective	(Yao et al., 2017b)
Nlrp3$^{-/-}$	AOM/DSS	Protective	(Zaki et al., 2010b, Allen et al., 2010)
Nlrp3$^{-/-}$	AOM/DSS	None	(Hu et al., 2011)
Nlrp3^{R258W}	AOM/DSS	Protective	(Yao et al., 2017b)

In murine of TNBS models the chemical compound is administered intrarectally and mediates a T cell-mediated immune response, similar to chronic colonic inflammation as described in CD (Wirtz and Neurath, 2007). In the DSS colitis model, DSS is dissolved in the drinking water and is ingested. The chemical causes significant damage to the epithelial barrier by causing acute colonic crypt destruction and mucosal ulceration. The compromised epithelial barrier is invaded by gut microflora which enters to the lamina propria resulting in massive infiltration of inflammatory cells and up-regulation of pro-inflammatory cytokines comparable to human ulcerative colitis (Chassaing et al., 2014, Ni et al., 1996). Administering DSS in weekly cycles alternating it with water results in a condition that is similar to clinically observed conditions of active and remission phases of UC. Finally, AOM a potent genotoxic carcinogen that causes DNA damage in epithelial cells is used in combination with repeated DSS administration. The resulting chronic inflammation promotes the development of colorectal cancer in cells carrying mutations generated by AOM (Tanaka et al., 2003).

The role of NLRP3 in colitis and CAC is still controversial with some studies showing a protective role while other studies demonstrate a detrimental effect of NLRP3 activation (Table 2.2). Studies of individual inflammasome components in colitis models before the inflammasome was identified as complex molecular platform questioned the role of caspase-1 (Dupaul-Chicoine et al., 2010, Siegmund et al., 2001b). The phenotype of *Caspase-1*$^{-/-}$ DSS colitis mice showed reduced acute and chronic colitis (Siegmund et al., 2001b). Both *Caspase-1*$^{-/-}$ and *Asc*$^{-/-}$ mice showed an increased susceptibility to DSS colitis and disease severity was linked to reduced IL-18 production. Conversely, the administration of exogenous IL-18 completely reversed severity of colitis (Dupaul-Chicoine et al., 2010). Intestinal epithelial cells rather than lamina propria cells were shown to be the source of IL-18 that was needed for early induction of tissue repair and epithelial cell regeneration.

The discrepancy in the above studies was explained by the novel discovery of two confounding aspects of the *Caspase-1*$^{-/-}$ mouse model. First, *Caspase-1*$^{-/-}$ mice additionally lacked caspase-11 (Kayagaki et al., 2011). Therefore, all results based on previous studies of this mouse model during DSS colitis is complicated by the contribution of caspase-11 to disease pathogenesis.

The second controversy revolves around the fact that the *Caspase-1*$^{-/-}$ *Caspase-1*$^{1129mt/129mt}$ mice harbour colitogenic microbiota that have shown to enhance the severity of DSS colitis (Elinav et al., 2011). A recent study characterised the intrinsic functions of caspase-1 *in vivo*. The study generated *Caspase-1*$^{-/-}$ and *Caspase-11*$^{-/-}$ mice on a pure C57BL/6N genetic background with a non-dysbiotic intestinal microbiota. Using these gut microbiota it could be shown that canonical caspase-1 activation, not caspase-11, is responsible for exacerbating DSS-induced colitis (Blazejewski et al., 2017).

The protective role of NLRP3 in colitis was suggested by experiments that showed enhanced colitis in *Nlrp3*$^{-/-}$ mice mediated by a loss of epithelial barrier integrity and reduction of IL-18 (Zaki et al., 2010a), defective antimicrobial mechanism leading to bacterial dysbiosis and increased susceptibility to DSS- and TNBS-induced colitis (Hirota et al., 2011).

The role of the NLRP3 inflammasome in the pathogenesis of UC was analysed in an oxazolone-induced murine colitis model. Intrarectal delivery of oxazolone (OXA) created a relevant UC pathogenesis model mediated by Th2 cytokines. In this model a reduction of mature IL-1β and IL-18 production induced a higher severity of colitis in OXA-treated *Nlrp3*$^{-/-}$ mice when compared to WT mice. Conversely this increase in severity could be prevented by exogenous administration of IL-1β or IL-18. This study shows that NLRP3 inflammasome-derived IL-1β and IL-18 may play a protective role against OXA-induced colitis (Itani et al., 2016).

Controversially, competing studies showed that DSS induced colitis was attenuated in *Nlrp3*$^{-/-}$ mice potentially mediated by a local reduction of pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ (Bauer et al., 2010). Exposure to DSS and concurrent treatment with the caspase-1 inhibitor Pralnacasan ameliorated colitis (Bauer et al., 2007). An attenuation of colitis in NLRP3-deficient mice has been confirmed by others supporting the function of NLRP3 as a negative regulator of the inflammatory process (Elinav et al., 2011).

Genetic models of *Nlrp3*$^{-/-}$, *Caspase-1*$^{-/-}$ and *Asc*$^{-/-}$ mice exposed to AOM/DSS have an increased tumour burden (Allen et al., 2010). Using bone marrow chimaeras, it was demonstrated that tumorigenesis suppressive signalling by NLRP3 was in the hematopoietic and not in the epithelial compartment. This correlated colitis-associated tumours with a defective production of IL-18 during the initiation of inflammation in the AOM/DSS model.

Consequently, the role of NLRP3 in CAC has mainly been reported as a negative regulator in colitis-associated tumorigenesis (Allen et al., 2010, Zaki et al., 2010b).

Consistent with a protective role of NLRP3 in tumorigenesis *Nlrp3*$^{-/-}$ mice were highly susceptible to AOM/DSS induced inflammation and the treatment caused increased tumours in the colon. The mechanism proposed was that NLRP3-dependent IL-18 secretion is required for STAT1 activation and IFN-γ induction leading to decreased immune tumour surveillance in the absence of IL-18. This phenotype was reduced with administration of recombinant IL-18 (Zaki et al., 2010b).

The ability of NLRP3 to release bioactive IL-18 is a potential mechanism explaining the protective role of this cytokine against CAC development. However, another study found no difference in CAC between NLRP3-deficient and WT mice in a AOM/DSS model (Hu et al., 2011). The reason for these discrepancies observed in the *Nlrp3*$^{-/-}$ colitis or CAC model phenotype is not clear but could be due to differences in length and concentration of DSS treatment or to baseline differences in the composition of the intestinal microbiota in experimental mouse colitis.

Human and murine studies suggest that bacterial dysbiosis promotes inflammation in human and mouse colitis and CAC models (Kitajima et al., 2001, Richard et al., 2018). Studies in mice deficient in inflammasome genes have provided evidence that specific inflammasomes like NLRP6 and NLRP3 are major regulators of commensal microbiota. A study in *Nlrp3*$^{-/-}$ mice suggested that the NLRP6/ASC inflammasome acts as a crucial regulator of the gut microbiota (Elinav et al., 2011). Another study that observed a significant increase in bacterial counts in stool, colon, mesenteric lymph node and liver in DSS colitis of *Nlrp3*$^{-/-}$ mice compared to DSS colitis in wild-type mice demonstrated that NLRP3 plays a role in controlling commensal overgrowth and bacteraemia (Zaki et al., 2010a).

How NLRP3 regulates the microbial composition in the intestines is not clear. This question was addressed by comparing faecal microbiota of *Nlrp3*$^{-/-}$ deficient mice to their WT littermates (Hirota et al., 2011). Only *Nlrp3*$^{-/-}$ mice were shown to carry a specific bacterial composition with potentially pathogenic members of the family Enterobacteriaceae including the species *Citrobacter, Proteus* and *Shigella* (Hirota et al., 2011). Furthermore, the unique genera *Mycobacterium, Collinsella, Clostridium* and

Ralstonia were identified which were not detectable in WT littermates. Additionally, it could be shown that colon crypt secretions obtained from *Nlrp3*$^{-/-}$ mice ex vivo had decreased bactericidal activity against *E. coli*. The significant difference in composition of the intestinal microbiota in the *Nlrp3*$^{-/-}$ mice could explain the increased susceptibility to DSS- and TNBS-induced colitis (Hirota et al., 2011).

Further studies have demonstrated that the distinct commensal bacterial species *Proteus mirabilis* induces a robust Il-1β secretion via NLRP3 inflammasome activation in newly recruited intestinal Ly6C^{high} monocytes during DSS colitis. This could be linked to an increased severity of disease (Seo et al., 2015). However, the molecular pathways by which intestinal bacterial populations in NLRP3 inflammasome-deficient mice promote intestinal inflammation are still elusive. It has been speculated that this could be due to their ability to upregulate the generation of pro-inflammatory chemokines and cytokines such as CCL5 (Elinav et al., 2011), IL-6 and TNF-α (Hu et al., 2013).

Nlrp3$^{-/-}$ mice show a more severe colitis when compared to WT mice after infection with the intestinal pathogen *Citrobacter rodentium* (Song-Zhao et al., 2013). This could be due to a lack of downstream cytokines IL-1β and IL-18 in the gene-deficient mice and allows the conclusion that activation of the NLRP3 inflammasome is necessary for an attenuation of *Citrobacter rodentium* driven intestinal inflammation.

In contrast to gene-deficient models, the *Nlrp3*^{R258W} mouse model displays an enhanced NLRP3 inflammasome signalling. These mice develop an auto-inflammatory response in the skin (Meng et al., 2009). In the intestine they maintain homeostasis and remain strongly resistant to experimental colitis and subsequently, colorectal cancer. This due to a distinct microbiota in *Nlrp3*^{R258W} mice with an increased presence of bacterial species such as *Clostridium XIVa* and *Lactobacillus murinus* and also a significant reduction of colitogenic bacteria such as *Akkermensia muciniphila* which promote the local differentiation of T_{regs}, that contribute to homeostasis in the gut (Yao et al., 2017b). Further work needs to be done to elucidate the signalling pathways by which resident bacteria stimulate the NLRP3 inflammasome to induce colitis in IBD.

2.7 Diet, microbiota and the activation of NLRP3: bowel cancer as an end stage of inflammation

Environmental factors, together with the individual genetic makeup and the innate immune response play role in development of IBD (Marion-Letellier et al., 2016). Two central environmental factors are diet and the composition of symbiotic microorganisms that live in our gastrointestinal tract, the so-called microbiome. Microbiota composition has been shown to modulate metabolism-associated conditions like obesity and inflammatory diseases of the bowel like IBD and associated cancers such as CAC and CRC (Requena et al., 2018).

A healthy diet helps to maintain a balanced and healthy microbiota and consequently, immune homeostasis in the gut. Unbalanced consumption of nutrients can lead to a dysbiosis of the microbiota and inflammation (Geuking et al., 2014, Marion-Letellier et al., 2016). Chronic insults from dietary metabolites activate NLRP3 and IL-1β production and thus progress disease pathogenesis (Camell et al., 2015). Consequently, diet has been identified as an important driver of the development a large percentage (50–90%) of tumours of the bowel (Kasdagly et al., 2014). For example, diet rich in fat increases the amount of bile acid which consists to 58% of deoxycholic acid (DCA). DCA has been found to disrupt epithelial integrity due to Cathepsin B activation of NLRP3 and leads to barrier disruption observed in DSS-induced colitis. In mice deficient for NLRP3 or caspase-1 DSS-induced colitis could not be established which underlines the role of NLPR3 in the development of colitis in a high fat environment (Zhao et al., 2016). Another chronic insult by the ingested diet is the presence of dietary cholesterol in the intestines. In an AOM-treated mouse model this has been associated with a hyperactivity of NLRP3 resulting in an increase in IL-1β (Du et al., 2016). In general, saturated lipids, ceramide (Vandanmagsar et al., 2011) and uric acid can act as DAMPs that induce unwanted NLRP3 activation by initiating the production of ROS (Camell et al., 2015).

On the other hand, some metabolites like omega-fatty acids prevent activation of NLRP3 and thus have an anti-inflammatory effect (Yan et al., 2013). These examples allow an insight how inflammation, colitis and CAC can be supported by various dietary parameters.

Under normal physiological conditions the symbiotic microbiota support the host by breaking down complex polysaccharides in dietary fibre into short chain fatty acids (SCFA) with less than six carbon atoms (Nieuwdorp et al., 2014). The predominant forms are acetate (C2), propionate (C3) and butyrate (C4). SCFA are the major products of bacterial fermentation and important as energy source for colonic epithelial cells (Huycke and Gaskins, 2004). SCFA induce a favourable intestinal environment in a variety of ways and high fibre diet boosts bacterial species which produce SCFA (Desai et al., 2016, Kelly et al., 2015). The presence of dietary fibre and SCFA also protects in DSS directly through activation of NLRP3 possibly in colonic epithelial cells (Macia et al., 2015). While the molecular pathways of this interaction are still elusive it demonstrates the important role of diet in intestinal homeostasis.

Furthermore, butyrate is one of the main SCFA induces functional T_{regs} via intrinsic up-regulation of the *Foxp3* gene (Furusawa et al., 2013). The extra thymic differentiation of T_{regs} in the periphery is enhanced via the conserved non-coding sequence 1 (CNS-1)-dependent pathway in the presence of butyrate and propionate (Arpaia et al., 2013). Butyrate activates the intestinal butyrate GPR109A receptor which drives the differentiation of T_{regs} and modulates immune responses by upregulation of the production of IL-10, suppressing colonic inflammation and carcinogenesis in a mouse model of intestinal inflammation associated with CAC (Singh et al., 2014). Increased T_{reg} initiate an anti-inflammatory response and act as antagonist to NLRP3 thus maintaining the homeostasis in experimental colitis and CAC (Yao et al., 2017b).

2.8 Future directions of colitis associated colon cancer therapy by intervention with NLRP3 activation

An improved understanding of the mechanistic interactions between diet and microbiota, and inflammasomes such as NLRP3 will reveal new potential therapies that target these pathways (Fig. 3). Here we outline some opportunities for interventions (Table 2.3).

Table 2.3: Opportunities for interventions with colitis and CAC

Potential NLRP3-mediated interventions to treat colitis and CAC	Therapeutic approach
Immunomodulators	Various dietary molecules which can help to reduce only detrimental activity of NLRP3
Stimulation therapy	Targeting the various activating mechanism of NLRP3
Inhibitory molecules	Designing inhibitory small molecules against activation, assembly or various subunits of NLRP3
Associated target therapy	Chemokines, cytokines, signalling cascade and immune cells like macrophage's which support in negative activity of NLRP3

2.8.1 Diet/metabolic immunomodulators

Metabolite targeted therapy can be used to modulate the hyper activation/blockage of NLRP3. Metabolic product induced inflammatory mediators like plasminogen activator inhibitor (PA)-1, sphingosine-1-phosphate and ceramide-1-phosphate constitute a link that allows modulation of NLRP3 activity. Targeting these mediators results in a therapy of colitis. Lipoxygenase is needed to recruit monosodium urate which activates NLRP3 in gout. This mechanism needs to be investigated in colitis and CAC models (Amaral et al., 2012). A further interesting possibility is the inhibition of ceramide synthesis by increasing fatty acid oxidation to reduce fatty acid inflammation (Schilling et al., 2013).

2.8.2 Inhibition of de novo activation of NLRP3

The cytoskeleton protein α-microtubulin is an activator of NLRP3. This protein supports NLRP3 assembly by recruiting ASC from mitochondria. This supporting role of NLRP3 activation requires an assistance from dynein (Akira et al., 2013). The microtubules have to undergo post-translational modification by acetylation by the *Mec17* gene product in order to activate NLRP3 (Akira et al., 2013). An activation of the enzyme SIRT2 which is α-tubulin deacetylase is required by DAMPs in order to activate NLRP3. A targeted therapy against this mechanism of NLRP3 assembly could offer an interesting treatment opportunity.

2.8.3 Small molecule targeted therapy

Novel small molecule specific inhibitors such as MCC950, CY-09, BHB has been used to pharmacologically target NLRP3 activation and successfully treat a variety of inflammatory disorders (Coll et al., 2015, Jiang et al., 2017, Youm et al., 2015). These inhibitory molecules target various protein components such as ASC or caspase-1 or inhibit production of NLRP3-mediated effector cytokines such as IL-1β (Mangan et al., 2018). At present there are no specific NLRP3 inhibitors used in clinical IBD therapy.

However, a few compounds that show inhibition of NLRP3 activity have emerged as potential therapeutics for IBD (Perera et al., 2017). Most of these are nonspecific with unknown mechanisms, limiting their progression to clinical usage for long term application in chronic IBD but specific NLRP3 inhibitors have shown promise in IBD patient tissues and colitis mouse models. A recent study using specific inhibitor MCC950 in the spontaneous chronic IBD mouse model Winnie, illustrated the potent therapeutic effect of NLRP3 blockade. Established colitis was ameliorated by a 3-week treatment of orally administered MCC950 (Perera et al., 2018). Another study used glyburide alleviating colitis and preventing disease onset in *Il10*$^{-/-}$ mice. This drug also inhibited pro-inflammatory cytokines in mucosal explants from Crohn's patients (Liu et al., 2016b).

Furthermore, the benzimidazole-containing synthetic small molecule inhibitor Fc11a-2 alleviated colitis in a DSS-induced colitis mouse model (Liu et al., 2013). In this study the mechanism of action was found to be inhibition of cleavage of pro-caspase-1 following a reduction in production of IL-1β and IL-18 suppressing the activation of NLRP3 inflammasome. Finally, a noncytotoxic, novel acrylate derivative inhibitor INF39 which does not block caspase-1.

Oral administration of INF39 in a TNBS-induced rat colitis model attenuated intestinal inflammation (Cocco et al. 2017). The use of small molecule inflammasome inhibitors also shows promise when it comes to prevention of CAC. A study on the small molecule andrographolide (Andro) shows that it is protective against AOM/DSS induced colon carcinogenesis in mice by inducing mitophagy in macrophages which leads to a reversed mitochondrial membrane potential collapse that inactivates the NLRP3 inflammasome and prevents the development of CAC (Guo et al., 2014).

A further example is the small-molecule AMPK activator GL-V9 which resolves colitis and is protective against tumorigenesis in colitis-associated colorectal cancer. GL-V9 acts by triggering autophagy in cells leading to activation of the NLRP3 inflammasome (Zhao et al., 2018). In summary the regulation of chronic inflammation in the intestines through pharmacological intervention of small molecule NLRP3 inhibitors in IBD patients could be a potential therapeutic option for preventing CAC.

2.8.4 Combination therapy

Combination of small molecule therapy along with diet modulators could be tried to increase effectiveness. For example, various combinations of diet modulators can be used such as 5-aminosalicylic acid together with fish oil. This combination lowered the inflammatory score compared to treatment with 5-aminosalicylic acid alone in rats with TNBS-induced colitis (Mbodji et al., 2013). In human tissues the combination of glutamine and arginine have been shown to reduce TNF-α production in colonic biopsies of colitis patients (Lecleire et al., 2008).

2.9 Conclusion

There is clear evidence supporting the NLRP3 inflammasome as key player in the inflammatory response in colitis (Schoultz et al., 2009, Villani et al., 2009) with ultimate implications for CAC development (Allen et al., 2010). However, it is also clear from the conflicting evidence outlined in this review that involvement of the NLRP3 inflammasome in inflammatory processes leading to tumour development is complex and may be context dependent. Therefore, carefully designed follow up experiments are warranted, where conditions for the induction of colitis including controlled intestinal microbiota, are carefully controlled. Unravelling the role of the NLRP3 inflammasome in intestinal inflammation will provide insights for the role of NLRP3 in intestinal epithelial cells and the pathways employed by the mucosal immune system to modulate the microbiota and integrate with the adaptive immune response to defend the integrity of the gut mucosa. Understanding the mechanisms that underpin these interactions also more broadly builds on the current strong interest in understanding the immune pathways underpinning chronic inflammation in tumorigenesis.

Chapter 2a

Introduction to Experimental Mouse models

2a.1 Spontaneous colitis mouse model Winnie

Experimental mouse models of colitis are usually induced by chemicals such as DSS or TNBS or pathogens such as nematodes (Brierley and Linden, 2014). However, these models do not resemble chronic intestinal inflammation as seen in human IBD. Thus, spontaneous chronic colitis models such as Winnie mice play an important role as experimental model for investigating IBD pathogenesis and therapy.

2a.1.1 Winnie mouse model generation

Winnie mouse model was derived from a from ENU (N-ethyl-N-nitrosourea) mutagenesis targeting a single missense mutation in the mucin 2 gene (*Muc2*) gene. Winnie and another mouse model Eeyore were the first animal models of spontaneous intestinal inflammation resulting from a single missense mutation in any gene. The single nucleotide polymorphism (SNP) is an alteration from a cysteine to tyrosine in the N-terminal D3 domain of *Muc2* of Winnie mice (C57BL/6 background). One significant feature of Winnie mice is that the mutation is confined to the intestinal primary epithelial and their underlying immune system is normal.

The gel forming mucin 2 is the major constituent of intestinal mucin responsible for its high viscosity and forming a protease-resistant matrix that retains molecules vital to host defence (*MUC2* and *Muc2* in humans and mice, respectively). *Muc2* is a glycoprotein macromolecule and is secreted by the goblet cells in the epithelial lining and released to the colonic mucus.

In Winnie mice, missense mutation in the *Muc2* gene resulted in the development of spontaneous colitis in which chronic intestinal inflammation resulted in 100% of the Winnie strain as early as within 6 weeks (Heazlewood et al., 2008). Investigations in to disease pathogenesis revealed that abnormal *Muc2* oligomerisation in endoplasmic reticulum (ER) resulted in unfolded-protein response (UPR). UPR caused ER stress in intestinal goblet cell resulting in cell defects and premature goblet cell apoptosis eventually leading to the development of chronic colitis spontaneously in Winnie (Heazlewood et al., 2008). Moreover, aberrant *Muc2* biosynthesis leads to depleted mucus barrier increasing

intestinal barrier permeability. The epithelial layer in normal conditions is protected by the sterile impenetrable inner mucus layer, however in Winnie mice the diminished mucus barrier exposed the epithelial layer to increased vulnerability to luminal bacteria, antigens and to inflammation-inducing toxins (Randall-Demllo et al., 2016).

2a.1.2 Winnie Clinical Symptoms

Winnie colitis closely represents the clinical symptoms representative of human UC including perianal bleeding, bloody stools, diarrhea, ulceration and weight loss (Heazlewood et al., 2008). Chronic inflammation in Winnie mice was established by detection of lipocalin-2 in faecal samples over a 4 week period (Robinson et al., 2016b). Another major feature of human UC seen in Winnie colitis is the chronic and relapsing nature of the disease.

Clinical symptoms appear at 6 weeks when animals reach adulthood and shows severe colitis at age of 16 weeks. In comparison to age matched C57BL/6 mice the proximal and distal colon were thickened, and colon weight was greater in Winnie. The spontaneous intestinal inflammation was most severe in the distal colon with age progressive disease severity, however this did not lead to growth retardation. Moreover, chronic inflammation did not develop spontaneous colonic tumours as seen in *Muc2*$^{-/-}$ mouse model (Velcich et al., 2002).

Winnie mice have been reported to be prone to developing rectal prolapse associated with intestinal inflammation. However, only 25% of the Winnie mice have been reported to develop rectal prolapse and although there has been no specific age reported to date, it could occur at any time between 9–20 weeks (Rahman et al., 2016).

2a.1.3 Winnie Histopathology

Histological evaluation of H&E-stained Winnie colonic sections showed aberrant crypt architecture, goblet cell loss, neutrophilic infiltration, crypt abscesses and focal epithelial erosions and ulceration particularly in the mid and distal colon. Consistent with observations from previously published studies Rahman et al, observed classical signs of colonic inflammation in Winnie due to thickening of muscle and mucosal layers, and increased CD45-immunoreactivity in the distal colon (Rahman et al., 2015b).

2a.1.4 Winnie immune profile

Extensive studies conducted in the past decade has detailed the immunological nature of spontaneous inflammation in Winnie mice. The original immunological description showed an increased local production of inflammatory cytokines such as TNF-α, IL-1β and IFN-γ in the distal colon explant cultures of Winnie mice. Increased production of T_H1 and T_H2 cytokines (TNF-α, IFN-γ, IL-13) was observed in *in vitro* MLN-derived leukocytes cultures. The pro-inflammatory cytokine IL-1β were elevated and increased in the intestines of both UC and CD patients and this was also observed with the Winnie colitis model (Heazlewood et al., 2008).

More detailed characterization showed a less secretion of the mucosal immune system conditioning factor TSLP (thymic stromal lymphopoietin). TSLP is a regulatory cytokine produced by intestinal epithelial cells and its role is suppressing dendritic cell activation, which is necessary for regulating immune responses to the normal gut microbiota. The decreased production of TSLP in Winnie results in an accumulation of activated intestinal lamina propria DC. These activated DCs produces IL-6 and IL-23 with a Th17 favouring chemokine milieu. Further investigations showed an elevated expression of *Th17* gene expression and associated cytokines such as IL-17 and IL-23 identified in Winnie mice demonstrates a dominant IL-23/Th17 immune response (Eri et al., 2011). The distal colon of Winnie mice featured increased levels of $CD11c^+$ APCs and activated dendritic cells DCs ($CD11c^+$ $MHC\text{-}11^{hi}$) which also assists in configuring a Th17-dominated mucosal immune response. The above observation may explain why the inflammation is most severe in Winnie distal colon that is also seen in human UC.

In summary, the immune profile of Winnie mice, DCs and macrophages, APCs, initiate an innate immune inflammatory response which is followed by adaptive immune responses which progress toward a mixed Th1, Th2 and a predominant Th17 mucosal immune response resulting in spontaneous colitis in a complex pattern as in human UC. The nature of the inflammatory response in Winnie mice has been extensively characterised (Figure 2a.1)

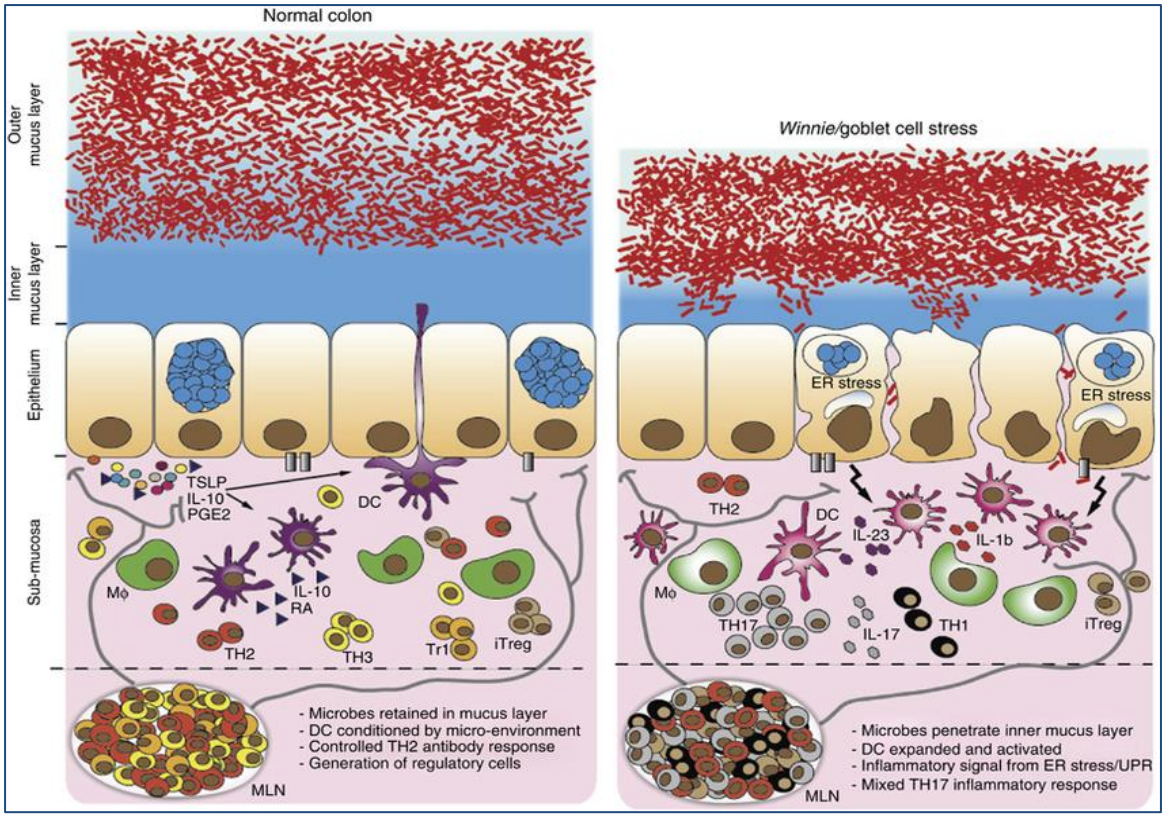

Figure 2a.1: Schematic representation of the initiation of inflammation in Winnie mice.
Reproduced from R.D. Eri et al (Eri et al., 2011).
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2a.1.5 Winnie intestinal innervation and colonic function

Enteric neurons and extrinsic nerves innervating the bowel have been suggested to play a role in the pathogenesis of IBD (Sharkey and Kroese, 2001). Histological evidence has shown an increased number of myenteric ganglion cells in chronic ulcerative colitis (Storsteen et al., 1953). The spontaneous chronic colitis in Winnie closely represents human IBD and therefore has been investigated to evaluate the intrinsic and extrinsic intestinal innervation especially of the distal colon. The distal colon of Winnie mice showed decreased density of sensory, cholinergic and noradrenergic fibres innervating the myenteric plexus, muscle and mucosa when compared to C57BL/6 mice. The innervation changes observed in Winnie mice were similar to changes observed in patients with ulcerative colitis (Rahman et al., 2015b).

In a separate study, the same group looked in to the innervation of the Winnie rectum and damage to enteric nervous system (ENS) associated with rectal prolapse. The study investigated changes in the myenteric and inhibitory motor neurons and compared changes

in the density of sensory afferent, sympathetic, and parasympathetic fibres in the rectum of prolapsed and normal Winnie mice. This study validated that Winnie prolapsed mice is associated with significant impairment of rectal innervation and muscular hypertrophy of the rectum (Rahman et al., 2016).

Further study by the same group investigated intestinal transit and colonic function of the Winnie colon (Robinson et al., 2017). Colonic transit was faster in *Winnie* mice while motility was altered including decreased frequency and increased speed of colonic migrating motor complexes and increased occurrence of short and fragmented contractions. Many of these changes are similar to disturbed motor functions seen in IBD patients.

2a.1.6 Winnie intestinal tumorigenesis

UC is also associated with an increased risk of colorectal cancer. however the underlying mechanisms have yet to be defined (Rutter et al., 2004). Randall-Demllo et al hypothesised that exacerbation of pre-existing chronic inflammation using colitogenic DSS in Winnie mice would induce colorectal tumorigenesis. Winnie mice exposed to three cycles of DSS displayed epithelial hyperplasia in the distal colon and crypt abnormalities resembling dysplasia. Tumour growth oncogenes *Cav1, Ccl5, Myc and Trp53* expression was differentially regulated in the distal colon of Winnie mice. However, no incident of colonic tumour growth was reported with DSS exacerbation of pre-existing chronic inflammation Winnie mice.

2a.1.7 Winnie Microbiota and Metabolomics

A recent study analysed the faecal microbial and metabolomic profiles in Winnie mice and discussed their relevance to human IBD (Robinson et al., 2016a). Composition of the dominant microbiota was found to be disturbed, and prominent differences were evident at all levels of the intestinal microbiome in faecal samples from Winnie mice, similar to observations in patients with IBD. In general, the abundance of members from the *Actinobacteria* and *Proteobacteria* was greater, whereas Bacteroidetes and *Tenericutes* were less present in samples from Winnie mice.

Analysing the metabolomics of faecal samples from Winnie determined changes in metabolites produced by the intestinal bacteria during colonic inflammation. Metabolic profile revealed that chronic colitis in Winnie mice associated with increases in CHO metabolites and decreases in amino acid metabolites, thereby generating disrupted CHO,

fat, and amino acid profiles in faecal samples. These results point to mechanisms of oxidative stress and malabsorption of nutrients in the colon, both of which are reported in IBD.

2a.1.8 Winnie a model for anti-colitis therapy

Winnie mice share similarities with human UC and has been used successfully as experimental model for investigating the effect of anti-colitis therapeutics. A study investigated the effect of the thiopurine 6TG on Winnie chronic colitis (Oancea et al., 2013) and found that the split dosing of 6TG was able to prevent the adverse thiopurine associated drug effect known as sinusoidal obstructive syndrome while preventing spontaneous colitis in Winnie mice.

Another study by the same group investigated the effect of glucocorticoid dexamethasone (DEX) in ameliorating ER stress in Winnie mice (Das et al., 2013). Winnie mice ER stress is caused by misfolding of the *Muc2* mucin resulting in UPR in goblet cells. DEX treatment in Winnie mice suppressed ER stress significantly restoring goblet cell *Muc2* production and ameliorating colitis. Therefore, glucocorticoids are potential drug in IBD, where they can reduce ER stress by promoting correct folding of secreted proteins and enhancing removal of misfolded proteins from the ER.

The Winnie mouse model was used to show the efficacy of IL-23 neutralizing antibodies in inflammatory bowel disease (Wang et al., 2015b). The results of the study show that neutralizing IL-23 using an anti-p19 antibody significantly downregulating TH17 proinflammatory cytokine expression and diminishing neutrophil infiltration alleviated both emerging and established colitis. Another result of this study supported clinical studies showing that IL-17 neutralization is not therapeutic in IBD.

In another study a novel mouse model was generated by crossing Winnie to understand the molecular mechanism underlying the anti-TNFα non-responding UC patient group (De Santis et al., 2017). The results demonstrated that IL-1β expression is a major pathway for the progression of colitis in TNFα-deficient Winnie colitis. These data also suggest that IL-1β can be a potential target for clinical intervention of UC patients who fail to respond to TNF-α neutralization.

We recently studied the effect of small molecule NLRP3 specific inhibitor MCC950 in the Winnie mouse model (Perera et al., 2018). The results illustrated that the efficacy of

MCC950 in the treatment of murine ulcerative colitis and provided avenue for a potential novel therapeutic agent for human inflammatory bowel diseases.

2a.2 Generation of Winnie x *Nlrp3*$^{-/-}$ mouse model

The Winnie mice used for this experiment was bred from an established strain at the University of Tasmania Cambridge Farm Facility. The *Nlrp3*$^{-/-}$ mice were imported directly to the Cambridge Farm Facility from University of Queensland, St Lucia, Australia.

In our animal facility, the imported *Nlrp3*$^{-/-}$ mice were rederived before being introduced into barrier housing facilities. Imported mice are always subjected to the rederivation procedure when procured from vendors or from other research labs which differ in veterinary health surveillance systems. The rederivation process is a standard procedure used to decontaminate certain pathogens prior to introducing them in to the colony (Sztein et al., 2011). Rederivation prevents possible outbreaks in the animal facility and misinterpretation of study results caused by infected animals. Rederivation also protects the staff and researches from the rare evet of receiving animals carrying a transmissible zoonotic disease.

For rederivation, embryos were harvested at preimplantation stage and were thoroughly washed for five times and surgically transferred into a clean Swiss Webster female mouse. At weaning, after the surrogate mice were screened negative for unacceptable pathogens, the pups were considered as pathogen-free to produce specific pathogen-free (SPF) mice suitable for breeding purposes. Based on the Mendelian pattern of inheritance a breeding plan was sketched to generate Winnie x *Nlrp3*$^{-/-}$ mice (Figure 2a.2). The first stage was where *Nlrp3*$^{-/-}$ mice were rederived by crossing with WT strain to produce 100% heterozygous *Nlrp3*$^{+/-}$ mice. Mice were then transported to the PC2 animal facility at the Menzies Institute for Medical Research, Hobart. In the second stage, heterozygous *Nlrp3*$^{+/-}$ mice were crossed with heterozygous *Nlrp3*$^{+/-}$ mice and were expected to produce 25% homozygous *(Nlrp3*$^{-/-}$*)*, 50% heterozygous *(Nlrp3*$^{+/-}$*)* and 25% wild type (WT). In the third step, Winnie homozygous mice were crossed with *Nlrp3* homozygous mice, and all pups produced were Winnie heterozygous and *Nlrp3* heterozygous. Subsequently, in the fourth step, Winnie heterozygous and heterozygous *Nlrp3* mice were crossed with Winnie heterozygous and heterozygous *Nlrp3* mice, resulting in the generation of 9 different genotypes (Table 2a.1). In this step, the Winnie x *Nlrp3*$^{-/-}$ experimental mice were

generated at 1:16 ratio as per Mendelian inheritance pattern. Many litters were produced until a working population of experimental mice resulted, and these were subsequently utilised for experimental purposes whilst maintaining healthy breeding pairs.

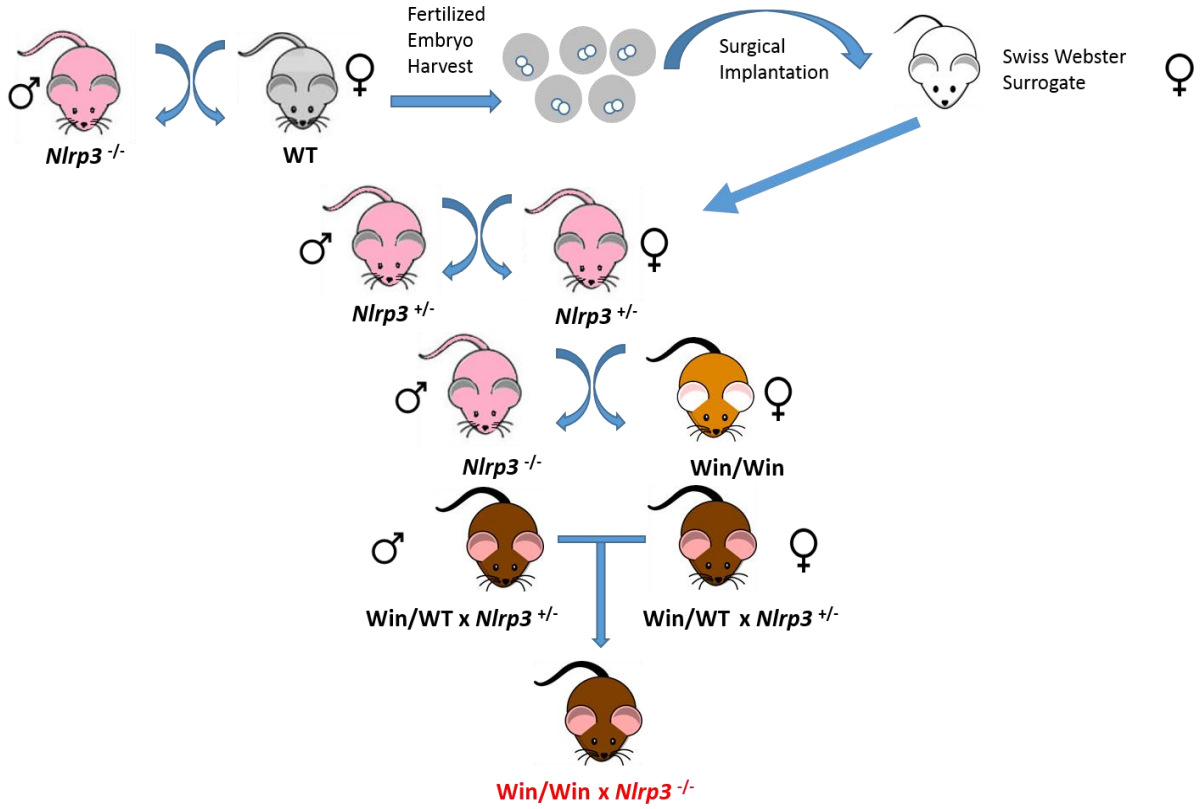


Figure 2a.2: Schematic presentation of generation of Winnie x *Nlrp3*$^{-/-}$ mice.

2a.3 Genotyping of experimental mouse models

Genotyping was performed for each batch of pups to determine the respective genotype. Optimisations of Winnie and *Nlrp3*$^{-/-}$ mice were performed until a working protocol was developed for the study. Appropriate genotyping assays were then performed and homozygous Winnie and *Nlrp3*$^{-/-}$ mice were identified.

2a.3.1 Extraction of DNA from mouse ear biopsies

The DNeasy Blood & Tissue Kit (69504, Qiagen) was used for genomic DNA (gDNA) extraction. Mouse-ear clips were lysed overnight using the supplied proteinase K and lysis buffer. Lysis was performed at 56°C with constant agitation at 500rpm using an Eppendorf ThermoMixer® C (5382000015, Eppendorf). Supernatant was then transferred to spin columns and processed according to the manufacturer's protocol and genomic DNA eluted (200µL). Neat DNA concentrations were used for PCR assays.

Table 2a.1: Efficient Generation of Winnie x *Nlrp3*$^{-/-}$ mice.

Stages	Parents		Offspring	Percentage
1	*Nlrp3*$^{-/-}$	WT	*Nlrp3*$^{+/-}$	100%
2	*Nlrp3*$^{+/-}$	*Nlrp3*$^{+/-}$	*Nlrp3*$^{-/-}$	25%
			Nlrp3$^{+/-}$	50%
			WT	25%
3	*Nlrp3*$^{-/-}$	Win/Win	Win/WT x *Nlrp3*$^{+/-}$	100%
4	Win/WT x *Nlrp3*$^{+/-}$	Win/WT x *Nlrp3*$^{+/-}$	WT/WT x *Nlrp3*$^{+/+}$	6.25%
			WT/WT x *Nlrp3*$^{+/-}$	12.5%
			WT/WT x *Nlrp3*$^{-/-}$	6.25%
			Win/WT x *Nlrp3*$^{+/+}$	12.5%
			Win/WT x *Nlrp3*$^{+/-}$	25%
			Win/WT x *Nlrp3*$^{-/-}$	12.5%
			Win/Win x *Nlrp3*$^{+/+}$	6.25%
			Win/Win x *Nlrp3*$^{+/-}$	12.5%
			Win/Win x *Nlrp3*$^{-/-}$	6.25%

2a.3.2 Winnie SNP genotyping assay

The presence of the Winnie *Muc2* mutation was confirmed using a custom-designed Winnie SNP PCR assay TaqMan probe where the reporter/quencher for Allele-1 was VIC/MGB-FAM, Allele 2- FAM/MGB-NFQ (Life Technologies, Assay ID: AHCSX8U, Catalogue No: 4332077, Size: 1500rxn, 40x). TaqMan genotyping master mix (4371355, Thermo Fisher Scientific) or TaqMan® GTXpress™ Master Mix (4401892, Thermo Fisher Scientific) was used for the PCR assay protocol detailed in Table 2a.2 and run in ABI StepOnePlus Real-Time PCR Systems (4376600, Applied Biosystems). The genotype of each mouse as identified by allelic discrimination plots seen in Figure 2a.3. Results of genotyping were analysed by using appropriate Step One software.

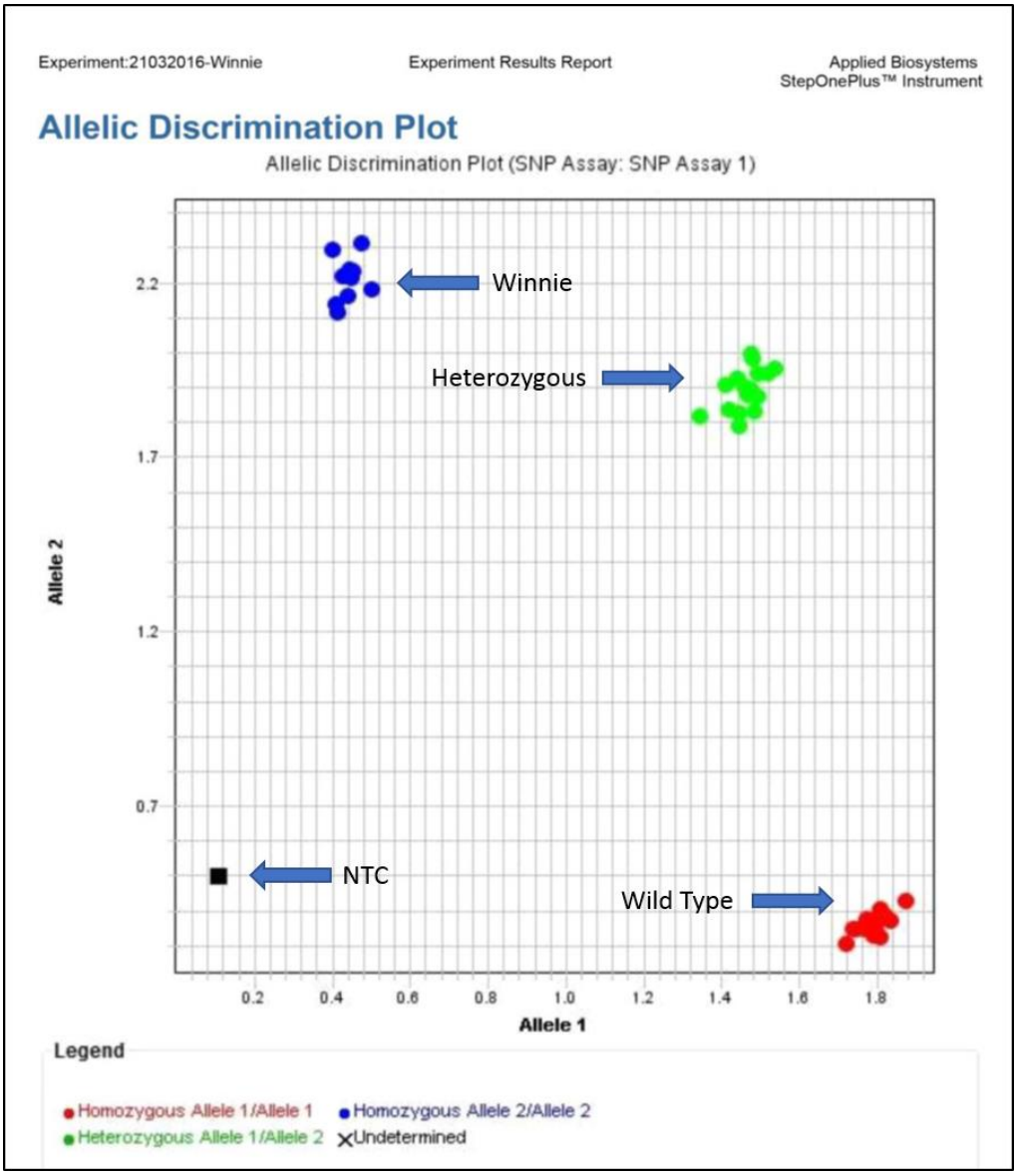


Figure 2a.3: Distribution of allelic discrimination plots of genotypes**.**

Allelic discrimination plot of SNP assay shows clusters of blue dots which represent Allele 2/Allele 2 (Win/Win) homozygous Winnie mutants. Allele 1/Allele 2 (Win/WT) heterozygous is represented as a green cluster of dots, and Allele 1/ Allele 1 wild-type (WT) is represented as red dots. No template control (NTC) is represented in black. The SNP assay was performed using neat samples and appropriate controls for each genotype.

Table 2a:2: Master Mix Preparation protocol

Reagents	**Volume per reaction**
2 x TaqMan Genotyping master mix	5 µl
20 x working stock of SNP	0.5 µl
DNase-free H_2O	3.5 µl
DNA Template (10ng/µl)	1 µl
Total Volume	10 µl

2a.3.3 *Nlrp3* genotyping assay

Deletion of the *Nlrp3* gene was confirmed (Table 2a.3) by traditional PCR with NLRP3 primers (20 µM) purchased from GeneWorks Pty Ltd. HotStar Taq Master Mix Kit (203445, Qiagen) was used with 2µl of genomic DNA in a reaction volume of 20 µl and thermal cycling performed according to program in Table 2a.4 using a thermal cycler (PTC-200, MJ Research).

The amplicons were run on an 1.2% agarose (A9539, Sigma-Aldrich) gel in 1x TBE (T4415, Sigma-Aldrich) and a DNA ladder (BIO-33045, Bioline) was used to identify the genotypes, and the size of fragments was 250 base pairs (bp) for wildtype allele and 500 base pairs for *Nlrp3* mutant allele (Figure 2a.4).

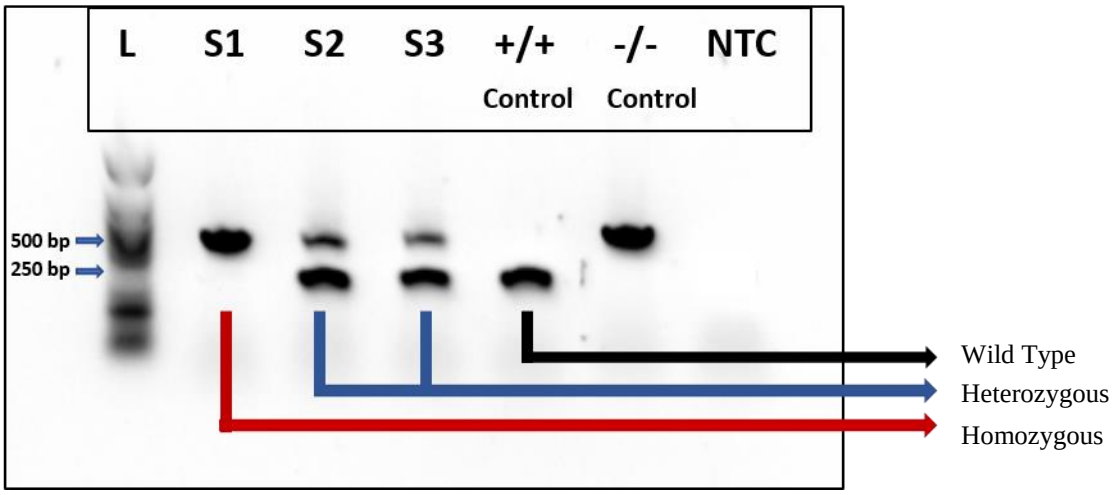


Figure 2a.4: Agarose Gel for *Nlrp3*$^{-/-}$ genotyping amplicons.

Agarose gel electrophoresis was performed, and the amplified fragments were visualised on 2% agarose (A9539, Sigma-Aldrich) gel using SYBR safe DNA gel stain (S33102, Thermo Fisher Scientific). A DNA easyladder 1 (BIO-33045, Bioline) was used, product

size band-100-2000 bp. The product size was 250 bp for homozygous *Nlrp3* and 500 bp for WT.

Table 2a.3: NLRP3 genotyping primer list

Primer	Sequence
Mutant	AAG TCG TGC TTC ATG T
WT Common	TCA AGC TAA GAG AAC TTT CTG
WT	ACA CTC GTC ATC TTC AGC A

Table 2a.4: NLRP3 genotyping thermocycle program

Primer	Temperature/Time	Cycles
Mutant	95°c/ 5 min	1x
WT Common	95°c/ 45 sec 95°c/ 30 sec 72°c/ 40sec	40x
WT	72°c/ 5 min	1x

Chapter 3

NLRP3 Inhibitors as potential therapeutic agents for treatment of Inflammatory Bowel Disease

3.1 Abstract

Inflammatory bowel disease (IBD) is a group of intestinal disorders characterised by chronic relapsing inflammation of the small intestine and colon. IBD manifests as either ulcerative colitis or Crohn's disease and increases the risk of developing colorectal cancer. Current treatment regimen for IBD utilise anti-inflammatory drugs, immune system suppressors and antibiotics or a combination of these. However, these therapeutics lead to several adverse effects, remission or significant non-responsiveness creating an urgent need to develop potent drugs with novel mechanisms of action for IBD. The inflammasome is a multiprotein complex that assembles to a key innate immune system signalling platform and is involved in the pathogenesis of inflammatory and autoimmune diseases. The NLRP3 inflammasome recognizes microbial and cell stress components and serve as a platform for caspase-1 activation and pro-inflammatory cytokine IL-1β, IL-18 maturation. Although the exact aetiology of IBD is unknown, uncontrolled NLRP3 Inflammasome activation has shown to play a major role in the chronic intestinal inflammation and mature IL-1β and IL-18 are consistently associated with increased colitis and colitis associated colorectal cancer development. In this review, we discuss the experimental NLRP3 inhibitors that have been investigated in IBD experimental models. The potential mechanism of action of these inhibitors such as inhibiting NF-κB activation and decreasing mitochondrial reactive oxygen species are discussed in detail. We further expand on the controversial role of NLRP3 in IBD and future issues that might arise from the long-term use of NLRP3 inhibitors in IBD therapy.

3.2 Introduction

Inflammatory bowel disease (IBD) is a group of intestinal disorders characterised by inflammation of the small intestine and colon. IBD is an important adolescent chronic disease, with 20–30% of patients presenting under 20 years of age (Kelsen and Baldassano,

2008). The incidence and prevalence of IBD is highest in the developed countries of North America and Europe (Bernstein et al., 2006, Munkholm et al., 1992). Approximately 2.5–3 million people in Europe suffer from IBD, with a direct healthcare cost of 4.6–5.6 bn Euros/year (Burisch et al., 2013).

The two major clinical types of IBD are Crohn's disease (CD) and ulcerative colitis (UC) (Hanauer, 2006). Both diseases are characterised by chronic series of relapses and remissions. Crohn's disease cause inflammation in all part of the intestinal tissue along the length of gastrointestinal tract, whereas ulcerative colitis is limited to the mucosal and submucosal layers of the colon and rectum. Common symptoms include diarrhoea with blood, abdominal pain, rectal bleeding and weight loss (Ponder and Long, 2013). Chronic gut inflammation is an important predisposing factor for the development of colorectal cancer (CRC) (Karin and Greten, 2005, Dupaul-Chicoine et al., 2010) with clinical data showing that UC increases cumulative risk of CRC by up to 18-20%, while CD is up to 8% after 30 years of disease (Rubin et al., 2012, Eaden et al., 2001, Canavan et al., 2006).

The standard therapy for IBD is reduction of symptoms and underlying inflammation by nonspecific anti-inflammatory corticosteroids (Fornaro et al., 2015). However the relief from corticosteroids is short lived and treatment disturb general immunity and can cause steroid dependency (Faubion et al., 2001). Infliximab is a novel monoclonal antibody targeting TNF-α which is efficient in inducing and preventing remission, however it can lead to serious side effects such as increased risk of opportunistic infection (Beaugerie et al., 2009). Moreover, significant number of patients are unresponsive to Infliximab (Naija et al., 2011).

Failure of current medical therapy and disease severity leads to colectomy which is an emergency surgery with a high rate of morbidity (Cima, 2010). To avoid surgery there is a need for developing next generation potent agents that target the underlying mechanisms driving the inflammation and regulate the long-term control of inflammation and maintain intestinal homeostasis.

IBD is thought to arise from dysfunctional activation of the intestinal mucosal immune system in response to commensal bacteria in a genetically susceptible individuals (Bouma and Strober, 2003). Although the exact aetiology of IBD has yet to be elucidated, recent studies support the hypothesis of a dysfunctional innate immunity as a major mechanism in chronic mucosal inflammation in IBD (Maloy and Powrie, 2011).

The innate immune response to cell stress or infection depends on the activation of receptors belonging to the pattern recognition receptors (PRRs) (Medzhitov and Janeway, 2002).The two main types of receptors are, Toll-like receptors (TLRs) and Nod-like receptors (NLRs) (Schroder and Tschopp, 2010, Martinon et al., 2009). Activation of these receptors initiate an immune response which involves activation of the inflammasome complex and a cascade of pro-inflammatory cytokines that initiates an adaptive immune response (Ranson and Eri, 2013). It is well known that the dysregulation of the inflammasome complex positively contributes to chronic inflammation and disease onset (Chen and Nunez, 2011, Elinav et al., 2013). Among the NLR activated inflammasomes, NOD-like receptor protein 3 (NLRP3) is the best characterized and most associated with inflammatory diseases such as atherosclerosis, type 2 diabetes gout, multiple sclerosis, Alzheimer's disease, Parkinson's disease, and age-related macular degeneration (Duewell et al., 2010, Guo et al., 2015a). Several reports have shown that the NLRP3 inflammasome plays a pathological role in experimental colitis (Bauer et al., 2012, Bauer et al., 2010). The NLRP3 inflammasome is a multimolecular platform constituting of NLRP3 protein encoded by the *Nlrp3* gene and an adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD) and procaspase-1. NLRP3 is expressed mostly in the cytosol of myeloid cells, lymphocytes, epithelial cells, and osteoblasts (Kummer et al., 2007).

NLRP3 inflammasome is activated by a range of pathogen-associated molecular patterns (PAMPS) and host derived damage-associated molecular patterns (DAMPS) released through cellular stress and damage. The known microbes that are recognized by NLRP3 includes *Candida albicans* (Gross et al., 2009), *Staphylococcus aureus* (McGilligan et al., 2013), *Escherichia coli* (Rathinam et al., 2012a), Influenza A virus (Allen et al., 2009). The endogenous host derived factors associated with damage are extracellular adenosine triphosphate (ATP) (Mariathasan et al., 2006), monosodium urate (MSU) crystals (Martinon et al., 2006), β-amyloid plaques (Halle et al., 2008) and islet amyloid polypeptide (Masters et al., 2010). Activation of the inflammasome leads to activation of caspase-1 and the consequent cleavage and secretion of mature IL-1β, IL-18 (Ranson and Eri, 2013) (Martinon et al., 2002) proinflammatory cytokines.

3.3 NLRP3 inflammasome: Novel Target for IBD

Many studies have investigated the role of NLRP3 inflammasome on intestinal inflammation in mouse models deficient in NLRP3 associated with enhanced susceptibility

to Dextran Sulphate Sodium (DSS). DSS a Sulphated Dextran with a high molecular weight, compromise the mucosal epithelial barrier function by increasing colonic mucosal permeability which leads to luminal antigens and microorganisms entering into the mucosa resulting in an overwhelming inflammatory response (Perše and Cerar, 2012). Administration of DSS leads to clinical observations similar to human UC, such as weight loss, diarrhea and occult blood in stool and anaemia. DSS treatment significantly increases expression of Th1 proinflammatory cytokines such as IL-12, IL-10, TNF-α, IL-1β and IFN-γ in the colon (Yan et al., 2009). Observational changes in the histology of the colonic mucosa with DSS treatment is increased inflammatory cell infiltration in to lamina propria and submucosa, mucin depletion, cryptitis and crypt abscesses. Acute colitis is induced by addition of 2–5% DSS to drinking water for an average of 7 days. Chronic colitis is induced by uninterrupted treatment of low concentrations of DSS or cyclical administration of DSS and water for up to 28 days.

Some studies indicate that *Nlrp3*$^{-/-}$ mice exhibited severe inflammation following oral DSS treatment (Dupaul-Chicoine et al., 2010, Zaki et al., 2010a) (Allen et al., 2010). In contrast to the above mentioned studies another study reported that the loss of NLRP3 or caspase-1 was protective in the DSS model of colitis and that *in vivo* inhibition of caspase-1 attenuated DSS-induced inflammation (Bauer et al., 2010). Due to contradictory findings the precise role of NLRP3 inflammasome on colitis is yet to be explained and warrants further experimental analysis.

However among the innate immunity inflammasomes, NLRP3 induced proinflammatory cytokines IL-1β and IL-18 has been consistently associated with promoting localized inflammation in IBD (Villani et al., 2009, Schoultz et al., 2009, McAlindon et al., 1998) and their correlation with disease activity (Guimbaud et al., 1998) have been well described. IL-1β and IL-18 are the major pro-inflammatory cytokines that promote activation of both the innate and adaptive immune responses (Dinarello, 2009, Chung et al., 2009). Moreover, IL-1β modulates the functions of dendritic cells, macrophages, neutrophils, as well as the differentiation of Th17 cells and can cause T cell mediated inflammation (Ziegler and Buckner, 2009, Coccia et al., 2012, Clambey et al., 2012). There is a strict correlation between the presence of IL-18 and the severity of inflammation (van de Veerdonk et al., 2011, Kanai et al., 2013). Furthermore, IL-18 neutralization (Siegmund et al., 2001a, Sivakumar et al., 2002) or chemical caspase-1 inhibitor (Bauer et al., 2007,

Loher et al., 2004) can effectively reduce severity in murine colitis. Therefore, inhibition of caspase-1 mediated IL-1β and IL-18 secretion may serve as a useful therapeutic option for patients with IBD (Coll et al., 2015, Thomas et al., 1991). Thus, targeting NLRP3 inflammasome complex appears to be a promising therapy option for IBD patients.

3.4 Promising NLRP3 inhibitors in experimental colitis and their mechanisms

NLRP3 inflammasome activation typically occurs in a two-step process (Sutterwala et al., 2006, Ozaki et al., 2015). The first priming signal leads to the activation of NF-κB mediated up regulation of the precursor proteins, pro-IL-1β, pro-IL-18, and NLRP3 (Franchi et al., 2012). In the second step, NLRP3 is stimulated by DAMPS and PAMPS which assembles NLRP3, ASC, and procaspase-1 components in to the NLRP3 inflammasome. The above complex initiates the conversion of procaspase-1 to caspase-1 leading to the production and secretion of mature IL-1β and IL-18 (Ozaki et al., 2015). Three different mechanistic pathways have been suggested for the above-mentioned conversion process. These are (1) pore formation and potassium efflux (Perregaux and Gabel, 1994, Petrilli et al., 2007), (2) lysosomal destabilization (Okada et al., 2014) and (3) mitochondrial reactive oxygen species (mROS) generation (Heid et al., 2013, Gurung et al., 2015). Given the evidence that aberrant NLRP3 activation is involved in the progression of IBD, targeting the activation pathway is a promising strategy for the development of novel effective therapeutics for IBD.

Since the discovery of the inflammasome (Martinon et al., 2002) many NLRP3 inhibitors have reached clinical trials and implemented as pharmacological agents in various inflammatory diseases. Examples include Canakinumab an IL-1β blocker, GSK1070806 an IL-18 blocker, Glyburide indirect NLRP3 inhibitor, Bay 11-7082 a NLRP3 ATPase inhibitor, Parthenolide a caspase-1 inhibitor and AZD9056 a P2X7 antagonists (Ozaki et al., 2015). However, these inhibitors are not specific to NLRP3 but disturb the signalling pathway or components of the inflammasome. Currently there are no specific NLRP3 inhibitors used in IBD therapy. Therefore, there is a great interest in the scientific community for the research of NLRP3 inhibitors for therapeutic use in IBD. We have summarised all the therapeutic agents and the mechanism of action in experimental IBD studies in Table 3.1.

Table 3.1: Novel experimental inhibitors of NLRP3 inflammasome in IBD.

Therapeutic Agent	Source	Experimental Model	Potential mechanism	Refs.
Alpinetin	Flavonoid	THP-1 cells DSS colitis mouse model	Blocking activation of NF-κB	(He et al., 2016a)
Wogonoside	Glucuronide	THP-1 cells DSS colitis mouse model	Blocking activation of NF-κB	(Sun et al., 2015)
MI-2 and mepazine	Small molecule MALT1 inhibitors	THP-1 cells DSS colitis mouse model	Blocking activation of NF-κB	(Liu et al., 2016b)
Fraxinellone	Lactone	THP-1 cells DSS colitis mouse model	Blocking activation of NF-κB	(Wu et al., 2014)
Dimethyl fumarate	Small molecule	THP-1 cells DSS colitis mouse model	Activating of Nrf2 to decrease mROS and mitochondrial DNA release	(Liu et al., 2016c)
MitoQ	Antioxidant	THP-1 cells DSS colitis mouse model	Decreasing mROS	(Dashdorj et al., 2013)
Asiatic acid	Plant isolate	THP-1 cells DSS colitis mouse model	Inhibiting mROS generation and mitochondrial dysfunction	(Guo et al., 2015c)
Andro	Small molecule	THP-1 cells DSS colitis mouse model	Triggering mitophagy for reversed mitochondrial membrane potential collapse	(Guo et al., 2014)
Fc11a-2	Synthetic small molecule	THP-1 cells DSS colitis mouse model	Inhibiting the cleavage of procaspase-1	(Liu et al., 2013)
HU 308	cannabinoid receptor 2 agonist	DSS colitis mouse model	Promoting autophagy and blocks NLRP3 activation	(Ke et al., 2016)
Fumigaclavine C	Alkaloidal metabolite of plant fungus	THP-1 cells DSS colitis mouse model	Unknown	(Guo et al., 2015b)
Magnesium lithospermate B	Plant isolate	DSS colitis mouse model	Unknown	(Jiang et al., 2016)
Apigenin	Phytoestrogen	DSS colitis mouse model	Unknown	(Marquez-Flores et al., 2016)
Astragalus polysaccharide	Plant isolate	DSS colitis mouse model	Unknown	(Tian et al., 2016)

The most common target of the experimental drugs, is inhibiting the activation of transcription factor NF-κB (He et al., 2016a, Sun et al., 2015, Liu et al., 2016b, Wu et al., 2014). The drug, Alpinetin is a plant flavonoid isolated from *Alpinia katsumadai* Hayata which can effectively inhibit the expression of TNF-α and IL-1β in DSS-induced mouse colitis. This study showed that pre-treatment with Alpinetin successfully reduced the levels of IL-1β in THP-1 cells, which was through inhibiting the expression of NLRP3

inflammasome complex. The mechanism was deduced to work through down-regulation of TLR4, NF-κ B and NLRP3 inflammasome (He et al., 2016a).

Wogonoside is another drug that targets NF-κB activation. This drug is a glucuronide metabolite of bioactive flavonoid wogonin with anti-cancer (Chen et al., 2013) and anti-inflammation-induced angiogenic activities (Chen et al., 2009). Wogonoside has shown to ameliorate clinical symptoms and histopathological features of DSS-induced colitis. Potential mechanism for Wogonoside is through inhibiting NF-κB activation leading to reduced formation of NLRP3 inflammasome and caspase-1 activity and thus suppressing IL-1β and IL-18 processing (Sun et al., 2015).

Another promising target predicts involvement of ROS, as many NLRP3 agonists such as ATP and MSU crystals have been shown to promote ROS formation (Petrilli et al., 2007, Cruz et al., 2007). The main source of cellular ROS is believed to be the mitochondria, and mitochondrial DNA (mt DNA) released by damaged mitochondria has also been shown to activate the NLRP3 inflammasome (Zhou et al., 2011). The electron transport chain in the mitochondrial inner membrane is critically involved in the generation of energy, where oxygen acts as an electron acceptor. When the electron transport chain breaks down, ROS can reach toxic levels within cells. Conversely, treatment of macrophages with the ROS inhibitors N-acetyl-L-cystine (Klionsky et al., 2012) or (2R,4R)-4- aminopyrrolidine-2,4-dicarboxylate (Dostert et al., 2008) has been shown to inhibit silica and asbestos-induced NLRP3 inflammasome activation.

MitoQ is a mitochondria-targeted antioxidant derived from ubiquinone, which is the active antioxidant component of coenzyme Q. The adsorbed MitoQ in the mitochondrial inner membrane acts as an antioxidant and is reduced by the respiratory chain to its active form and protects mitochondria from oxidative damage. MitoQ demonstrates a good pharmacokinetic profile and has been successfully trialled as a daily oral tablet in Parkinson's disease patients for a year in phase 2 trials (Snow et al., 2010). *In vivo* and *in vitro* studies have shown MitoQ to have good anti-oxidative, anti-inflammatory and anti-apoptotic effects in many oxidative damage-related pathologies (Graham et al., 2009). MitoQ was investigated as a potential therapeutic agent to treat acute colonic injury in a mouse model of DSS-induced colitis (Dashdorj et al., 2013). Results show that MitoQ was able to reduce oxidative stress and restore mitochondrial alterations and thereby decrease the excessive activation of the NLRP3 inflammasome leading to significant improvement

in clinical and histological changes seen in the DSS induced mouse model of colitis. These results suggest that mROS may play an important role in IBD and indicate that MitoQ could be a promising candidate for treatment of human IBD.

The experimental drug Dimethyl fumarate (DMF) dose-dependently attenuated body weight loss, colon length shortening and colonic pathological damage including decreased myeloperoxidase (MPO) and inducible nitric oxide synthase (iNOS) activities in Dextran Sulphate Sodium (DSS)-induced mouse model (Liu et al., 2016c). Furthermore, protein and mRNA levels of pro-inflammatory cytokines, including IL-1β, TNF-α and IL-6 were markedly suppressed by DMF. At the same time, decreased activation of caspase-1 was detected in DMF-treated mice, indicating that the NLRP3 inflammasome activation was suppressed. Nuclear factor-erythroid 2-related factor 2 (NRF2) is a redox-sensitive transcription factor that regulates multiple anti-oxidative enzymes (Suzuki et al., 2013). NRF2 is indicated in the protection against many inflammatory diseases (Li et al., 2008).The inhibitory mechanism was attributed to the up regulation of NRF2 which decreased mROS generation and mtDNA release.

MCC950 is a small molecule NLRP3 inhibitor that has gained immense impact in the research community in recent times. MCC950 is a proven highly selective and potent inhibitor of the NLRP3 inflammasome (Coll et al., 2015). MCC950 block NLRP3-dependent inflammasome activation at nanomolar concentrations, with no effect on NLRC4, NLRP1 or AIM2 inflammasomes. *In vivo*, MCC950 reduced IL-1β production and attenuated the severity of experimental autoimmune encephalomyelitis, a disease model of multiple sclerosis which is known to be aggravated by the NLRP3 inflammasome (Gris et al., 2010). MCC950 was effective in preventing the neonatal lethality in a mouse model of cryopyrin-associated periodic syndromes whereas blockade of IL-1β did not prevent lethality. This suggest that inflammasome inhibitors are more potent than the inhibition of IL-1β alone in ameliorating inflammatory damage. The study provided a detailed pharmacokinetic profile of MCC950 even though the mechanism of action was not resolved. This invaluable data has made it possible for this drug to be applied as a therapeutic agent in other inflammatory diseases such as myocardial infarction (van Hout et al., 2016) and kidney fibrosis (Ludwig-Portugall et al., 2016). MCC950 is an ideal candidate to be used as a therapeutic agent for the selective inhibition of NLRP3 in IBD.

NIMA-related kinase 7 (NEK7) is a member of the NIMA (never in mitosis gene A) related serine-threonine kinase family and is known to be involved in mitotic progression downstream of NEK9 (Belham et al., 2003). In a major breakthrough of the NLPR3 activation process two recent independent studies have concluded that NEK7 is necessary for the activation of the NLRP3 complex (He et al., 2016b). NLRP3 inflammasome formation is dependent on NEK7 binding directly to the LRR domain of NLRP3 downstream of the generation of mROS. The NEK7 role as a regulator of NLRP3 activation can be a novel promising therapeutic target for managing NLRP3 associated inflammatory diseases by targeting NEK7 to specifically inhibit NLRP3 inflammasome.

3.5 NLRP3 inhibitors: A double edged sword?

The NLRP3 inhibitors for IBD are still at the initial stage of drug development of biological confirmation in cellular and animal models (Table 1). Caution should be exercised when these drugs progress on for human clinical trials due to the controversial role of NLRP3 inflammasome and blockade of IL-1β and IL-18 cytokines in IBD.

The functions of IL-18 are complex and their possible contribution to chronic inflammation in the intestine is unclear (Siegmund, 2010b). Polymorphism in the gene encoding IL-18RAP has been associated with IBD (Zhernakova et al., 2008) while other studies reported that *Il-18*$^{-/-}$ or *Il-18R*$^{-/-}$ mice are more susceptible rather than resistant to DSS-induced colitis (Takagi et al., 2003, Reuter and Pizarro, 2004). A study on mice deficient for NLRP3 shows that they are more vulnerable to DSS-induced colitis due to lack of IL-18 and not IL-1β (Hirota et al., 2011). Mice deficient in caspase-1 are also more vulnerable to DSS-induced colitis, and administration of IL-18 initiates mucosal healing in these mice (Dupaul-Chicoine et al., 2010). These studies show a protective role for IL-18 possibly due to it being continuously expressed in intestinal epithelial cells and its role in maintaining the intestinal barrier under homeostatic conditions. Therefore, inhibiting NLRP3 will drastically inhibit IL-18 production and this could exacerbate IBD.

NLRP3 inflammasome has been shown to be responsible for protection against increased tumorigenesis (Allen et al., 2010). NLRP3 inflammasomes anti-carcinogenic capability is by inhibiting cell proliferation and promoting pyroptotic cell death pathways (Salaro et al., 2016, Dupaul-Chicoine et al., 2015). Therefore, NLRP3 inflammasome is essential for the homeostasis of epithelial barrier function and wound repair processes during IBD. This

observation suggests that elevated inflammatory responses and increased destruction of the epithelial barrier in the absence of a functional NLRP3 inflammasome drives colitis-associated tumorigenesis. Therefore, there is a possibility that the usage of NLRP3 inhibitors in IBD patients could lead to the development of CRC.

IBD is known to lead to extra-colonic inflammatory manifestations in other tissues such as lungs, joints, eyes, skin and liver (Levine and Burakoff, 2011). The physiological function of NLRP3 in these systems could be different to NLRP3 role in the intestine. Therefore, it is extremely important to evaluate the pharmacokinetics and long-term effect of these novel NLRP3 inhibitors designed for IBD before progressing to human clinical trials.

Majority of the studies of the efficacy of NLRP3 inhibitors detailed in Table 3.1 have used DSS induced colitis as the experimental model. DSS was given for up to 7 days to induce an acute colitis condition and treatment for less than 30 days. However, IBD is a chronic disease with long term use of therapeutic drugs. Therefore, the experimental conditions in these studies are not translational to IBD conditions and the efficacy of the inhibitors are uncertain. It would be more reliable to design experiments in a spontaneous chronic colitis murine model (Eri et al., 2012) that mimics human IBD condition to evaluate the effect of novel NLRP3 inhibitors.

3.6 Conclusion

Taken together, all the NLRP3 inhibitors showed significant ability to inhibit NLRP3 inflammasome activation and significantly improved clinical and histological changes in the DSS induced mouse model of colitis. All the inhibitors show potential use in the treatment of inflammatory bowel diseases. However, NLRP3 function is controversial at the moment with protective and harmful effects observed in murine colitis models. Therefore, when considering applying NLRP3 inhibitors for long term therapeutic relief for a chronic disease such as IBD more research needs to be done to analyse the complete structure and physiological function of NLRP3. The next exciting step would be to investigate if the protection offered in murine colitis models could be replicated when the inhibitors are applied to human IBD clinical trials.

Chapter 4

MCC950, a specific small molecule inhibitor of NLRP3 inflammasome attenuates colonic inflammation in spontaneous colitis mice

4.1 Abstract

MCC950 a potent, highly specific small molecule inhibitor of canonical and noncanonical activation of NLRP3 inflammasome has been evaluated in a multitude of NLRP3 driven inflammatory diseases. However, the effect of MCC950 on colonic inflammation has not yet been reported. In the present study we investigated the effect of MCC950 in a spontaneous chronic colitis mouse model Winnie, which mimics human ulcerative colitis. Oral administration of 40 mg/kg MCC950 commencing at Winnie week seven for three weeks significantly improved body weight gain, colon length, colon weight to body weight ratio, disease activity index and histopathological scores. MCC950 significantly suppressed release of proinflammatory cytokines IL-1β, IL-18, IL1-α, IFN-γ, TNF-α, IL-6, IL17, chemokine MIP1a and Nitric Oxide in colonic explants. Moreover, MCC950 resulted in a significant decrease of IL-1β release and activation of caspase-1 in colonic explants and macrophage cells isolated from Winnie. Complete inhibition with MCC950 in Winnie colonic explants shows, for the first time, the contribution of inflammatory effects resulting exclusively from canonical and noncanonical NLRP3 inflammasome activation in colitis. Taken together, the results illustrate the efficacy of MCC950 in the treatment of murine ulcerative colitis and provides avenue for a potential novel therapeutic agent for human inflammatory bowel diseases.

4.2 Introduction

Inflammatory bowel disease (IBD) is a group of intestinal disorders characterised by inflammation of the gastrointestinal tract (Ponder and Long, 2013). The two major types of IBD are Crohn's disease and ulcerative colitis. Crohn's disease causes inflammation in all parts of the intestinal tissue along the length of gastrointestinal tract while ulcerative colitis is restricted to the mucosa of the colon and rectum. Both diseases are characterised by a series of relapses and remissions and also increases the risk of colon cancer (Terzić et al.,

2010). The aetiology and pathogenesis of IBD is still unclear. Emerging evidence support the hypothesis that the dynamic key players are dysbiosis in enteric microbiota, a dysfunctional epithelial barrier, and defective innate immunity (Elinav et al., 2013).

The innate immune response to cell stress or infection depends on receptors such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs) (Schroder and Tschopp, 2010, Martinon et al., 2009). In particular NLRP3 is one of the best characterized and is associated with inflammatory diseases (Duewell et al., 2010, Rajamaki et al., 2010). The NLRP3 inflammasome is a cytoplasmic multimolecular platform composed of NLRP3 protein bound to an adaptor protein, apoptosis-associated speck-like protein containing a CARD (ASC) and procaspase-1. Activation of the inflammasome leads to proteolytic activation of caspase-1 triggering cleavage and subsequent secretion of proinflammatory cytokines IL-1β and IL-18 (Martinon et al., 2002). Kayagaki et al., 2011 described a novel non-canonical pathway resulting in NLRP3 inflammasome activation. This pathway is via caspase-11, which is widely expressed in both hematopoietic and non-hematopoietic cells, including macrophages and epithelial cells. Caspase-11 is activated by cytosolic gram-negative bacteria leading to pyroptosis and IL-1α and HMGB1 release, and NLRP3 inflammasome assembly and maturation of IL-1β, IL-18 (Kayagaki et al., 2011).

IL-1β cytokine levels are significantly altered in patients suffering from either acute or chronic gastrointestinal inflammation and have been additionally implicated in tumour angiogenesis, progression, and metastasis (Bioque et al., 1995, Casini-Raggi et al., 1995). Many clinical studies show evidence of increased IL-1β secretion from colonic tissues and macrophages of IBD patients, correlating to the severity of disease (Coccia et al., 2012, McAlindon et al., 1998, Ligumsky et al., 1990). Preclinical studies imply that IL-18 contributes the pathogenesis of colitis (Kanai et al., 2013, van de Veerdonk et al., 2011). Moreover, IL-18 neutralization (Siegmund et al., 2001a, Sivakumar et al., 2002) or pralnacasan inhibition of caspase-1 (Bauer et al., 2007, Loher et al., 2004) effectively reduced severity in murine colitis. These clinical findings suggest IL-1β and IL-18 play an important role in the pathogenesis of IBD.

A study by Bauer et al., 2010 found that NLRP3-deficient mice were significantly protected from colitis in DSS-induced colitis mouse model (Bauer et al., 2010) suggesting that the blockade of NLRP3 inflammasome may serve as a potential target for the development of novel therapeutics for patients with colitis. However, current pharmacological modulators

of NLRP3 inflammasome tested in experimental colitis are not specific to NLRP3 inflammasome and do not inactivate both canonical and noncanonical pathways (Perera et al., 2017).

MCC950 is a potent highly specific small molecule inhibitor of both canonical and noncanonical activation of NLRP3 inflammasome. *In vivo*, MCC950 reduced IL-1β production and attenuated the severity of experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis which is known to be aggravated by the NLRP3 inflammasome (Coll et al., 2015). Inhibition of NLRP3 by MCC950 effectively rescued neonatal lethality in a mouse model of cryopyrin-associated periodic syndrome, a genetic disease caused by activating mutation in NLRP3. In agreement with cell profiling, MCC950 was not effective against an NLRP1 mutant highlighting the compounds specificity *in vivo*. The study provided a detailed pharmacokinetic profile of MCC950 but the mechanism of action was elusive; MCC950 did not affect K^+ efflux, Ca^{2+} flux, NLRP3-NLRP3 or NLRP3-ASC interactions (Coll et al., 2015). Further work by Primiano et al., 2016 (Primiano et al., 2016), dismissed other likely targets of MCC950 such as GST Omega 1-1 (Laliberte et al., 2003), SUR1, SUR2a and SUR2b. Moreover MCC950 did not target cellular proteins involved in the activation of NLRP3 inflammasome such as caspase-1, SYK, JNK, GPR40, and GPR120 (Primiano et al., 2016). Only very weak off-target activity was identified through multiple commercially available screening panels (Eurofins Cerep, DiscoverX, Reaction Biology, Carna Biosciences, WuXi AppTec) (Primiano et al., 2016). In 2016 two independent studies discovered NEK7, a serine-threonine kinase, as an upstream regulator of NLRP3 inflammasome activation (He et al., 2016b, Shi et al., 2016). This major discovery of a new inflammasome component, revealed a potential therapeutic target for the inhibitory mechanism of sulfonylurea molecules such as MCC950 and glyburide. We and others hypothesise that MCC950 and glyburides target of inhibition could be the NEK7-NLRP3 interaction (Van Hauwermeiren and Lamkanfi, 2016).

MCC950 is the most specific and well characterised NLRP3 inhibitor known to date and has been tested in a diverse array of NLRP3 engaged inflammatory diseases. MCC950 shows promising therapeutic potential for reducing crystal-induced kidney fibrosis in mice (Ludwig-Portugall et al., 2016), reversing inflammation and blood pressure in a hypertension mouse model (Krishnan et al., 2016), in valosin-containing protein associated disease (Nalbandian et al., 2017) and decreasing inflammation associated with pathogenic

Influenza A Virus (Pinar et al., 2017). Temporal administration of MCC950 was able to reduce lung inflammation and cellular influx (Tate et al., 2016). However, MCC950 was not effective in reducing angiotensin II induced hypertension (Dinh et al., 2017) and in the treatment of acute procedural inflammation in burn-injured mice (Deuis et al., 2017). This was however due to the limited role of NLRP3 inflammasome in these disease models.

Recently MCC950 was recommended as an ideal therapeutic candidate for the selective inhibition of NLRP3 in colitis (Perera et al., 2017). Pellegrini et al,.2017 suggested MCC950 treatment will define anti-inflammatory effects resulting exclusively from inhibition of canonical and noncanonical NLRP3 inflammasome activation in colitis (Pellegrini et al., 2017). At present, the majority of available studies on the efficacy of NLRP3 inhibitors have used Dextran Sulphate Sodium (DSS) induced acute colitis as the experimental model of ulcerative colitis (Perera et al., 2017). The DSS colitis model is very established due to its rapidity, reproducibility and controllability. The DSS chemical exerts a toxic effect on colonic epithelium leading to a leaky tight junction and bacterial translocation (Poritz et al., 2007). Therefore it is reflective more of an acute injury than an inflammatory disease (Ni et al., 1996). In addition, DSS induced colitis development does not involve the T and B cell immunity which is unlike human ulcerative colitis (Chassaing et al., 2014). Due to these limitation in DSS induced colitis there is a great need for clinically relevant spontaneous colitis murine models which resembles human disease for understanding the inflammatory immune process of ulcerative colitis.

In this study we have used the spontaneous chronic colitis mouse model Winnie which develops spontaneous distal intestinal inflammation as early as 6 weeks of age and progresses over time to severe colitis by 16 weeks (Eri et al., 2011, McGuckin et al., 2011). Chronic colitis in Winnie is due to a primary epithelial cell defect due to a point mutation in the *Muc2* mucin gene resulting in aberrant *Muc2* biosynthesis leading to endoplasmic reticulum stress in intestinal goblet cells and reduced secretion of mucus which is very similar to active ulcerative colitis in humans (Pullan et al., 1994) (Heazlewood et al., 2008). Winnie mice display symptoms of diarrhoea, ulcerations, rectal bleeding and pain at different stages of colitis similar to human disease. Extensive studies done in Winnie has proven it to be the best available murine model to study human chronic colitis and its pathogenesis (Rahman et al., 2015a, Robinson et al., 2017, Robinson et al., 2016a). The

aim of this study was to investigate the therapeutic effect of MCC950 on Winnie and the results show a significant reduction of colitis.

4.3 Methods

Animals

All animal experiments were approved by the Animal Ethics Committee of the University of Tasmania (Ethics approval number: A16166) and conducted in accordance with the Australian Code of Practice for Care and Use of Animals for Scientific Purposes (8th Edition 2013). Mice were housed in a temperature-controlled environment with a 12-hour day/night light cycle. Individual body weights were assessed daily over an initial acclimation period of 7 days. All mice had access to radiation-sterilised rodent feed (Barastoc Rat and Mouse, Ridley AgProducts, Australia) and autoclaved tap water for drinking ad libitum during experiments. All efforts were made to minimize animals' suffering and to reduce the number of animals used.

Explant Culture

Mice were euthanized by CO_2 asphyxiation. The colons were dissected and removed from C57BL/6 and Winnie mice (n=5, 12 weeks). The colon was opened, and the faecal matter removed and cut in half lengthways, sectioned into distal and proximal tissue and weighed. Tissues are washed in PBS (P3813, Sigma-Aldrich) containing 1% penicillin/streptomycin (10000 U/ml) (1% P/S) (15140122, Gibco) three times. Equivalent amount of tissue was placed in a 24 well cell culture plate in growth media RPMI 1640 (11875085, Gibco) supplemented with 10% FCS (16000044), 1% P/S (15140122, Gibco). The tissues were stimulated with 10 ng/ml Lipopolysaccharide (LPS) from *Escherichia coli* serotype EH100 (ra) TLRgrade (ALX-581-010-L001, Enzo Life Sciences) for 2 hours. The medium was removed and replaced with serum-free medium (SFM) containing MCC950 (0.001-10 μM) (AG-CR1-3615, Adipogen), glyburide (200 μM) (10238-21-8, Sigma-Aldrich) and incubated for 24 hours at 37 ^{0}C in a moist atmosphere of 5% CO_2. After which the supernatants were removed and centrifuged at 12,000 g at 4 ^{0}C for 15 min and stored at -80 ^{0}C for cytokine analysis. Tissue was stored in RIPA buffer (R0278, Sigma-Aldrich) with protease inhibitor (4693116001, Sigma-Aldrich) to be analysed by Western blot. Supernatants were assayed for cytokine levels by ELISA kits according to the manufacturer's instructions IL-1β (DuoSet, R&D Systems), IL-10 (BMS614-2,

Invitrogen), TNF-α (KMC3011 Invitrogen) and concentrations were normalized to the weight of the explants.

Isolation of murine macrophages

Mice were euthanized by CO_2 asphyxiation. Peritoneal macrophages (IP) were isolated from the peritoneal cavity of C57BL/6 and Winnie mice (n=4, 12 weeks) by injection of 10 ml of PBS (P3813, Sigma-Aldrich). After 30 seconds of abdominal massaging, peritoneal lavage was performed. Collected peritoneal lavage was washed twice in PBS (P3813, Sigma-Aldrich) and plated in 6 well plates suspended in RPMI-1640 (11875093, Gibco) medium containing 10% FCS (10437-028, Gibco) 1% P/S (15140122, Gibco) for two hours. Non-adherent cells were removed by washing the plate twice with PBS (P3813, Sigma-Aldrich). The adherent macrophages were analysed in subsequent experiments.

Bone marrow derived macrophages (BMDM) cells were isolated from tibiae and femurs of C57BL/6 mice and Winnie mice (n=4, 12 weeks) and cultured suspended in RPMI-1640 (Gibco 11875093) medium containing 10% FCS (Gibco, 10437-028), 1% P/S (15140122, Gibco) and 10 ng/ml human macrophage colony stimulating factor (M-CSF) (130-094-129, Miltenyi Biotec). Culture medium was exchanged every 3 days. Under these conditions, an adherent macrophage monolayer was obtained at 7-8 days. Cells were harvested and seeded on 6-well plates. After culturing for 6 hours without M-CSF (130-094-129, Miltenyi Biotec), the cells were used for the experiments as BMDM.

For the isolation of Mesenteric lymph node (MLN) macrophages the peritoneal cavity of Winnie (n=4, 12 weeks) was opened and the gut was taken out so that the MLN were visible. The MLN were excised and placed in chilled PBS (P3813, Sigma-Aldrich). To generate a single cell suspension, the MLN were placed on a sterile 70 μm nylon mesh cell strainer (22-363-548, Fisher brand) and was mechanically disrupted into the mesh using the base of a plunger from a 1 cc syringe. Cells were washed in PBS (P3813, Sigma-Aldrich) containing 1% FCS (10437-028, Gibco). Cell suspension was decanted through a second 70 μm cell strainer (22-363-548, Fisher brand) to remove any remaining cellular aggregates or tissue debris. Cells were subjected to gentle centrifugation at 500 g for 5 min. Supernatant was decanted and cells re-suspended and cultured in RPMI-1640 medium (11875093, Gibco) containing 10% FCS (10437-028, Gibco) and 1% P/S (10437-028, Gibco) till an adherent macrophage monolayer was obtained.

Single cell LPMC (lamina propria mononuclear cell) suspensions were prepared from 12-week-old Winnie n=12 mice. The colons were dissected carefully and washed with ice-cold PBS (P3813, Sigma-Aldrich) and cut in to small pieces. Fragments were placed in Hanks' balanced salt solution (H9269, Sigma-Aldrich) containing 5 mM EDTA (E6758, Sigma-Aldrich) and 1 mM DTT (10197777001, Sigma-Aldrich) 37 °C for 40 mins with gentle shaking to remove the epithelial layer. The colon segments were then digested in PBS (P3813, Sigma-Aldrich) containing 0.5 mg/mL collagenase (C2139, Sigma-Aldrich), 0.5mg/mL DNaseI (11284932001, Roche) and 3 mg/mL Dispase II (D4693, Sigma-Aldrich) at 37°C, at slow rotation, for 1.5 hours. Supernatants were collected by filtering through a 70 μm cell strainer (22-363-548, Fisher brand). Filtered cells were layered on a 40/80 Percoll gradient (P4937) and centrifuged at 1000 ×g for 20 min. The separated LPMCs were washed twice, and re-suspended and cultured in RPMI-1640 medium (Gibco 11875093) containing 10% FCS (10437-028, Gibco) and 1% P/S (10437-028, Gibco).

Cell viability assay

Cell viability of BMDMs was measured using alamarBlue® reagent (A50101, ThermoFisher Scientific). Briefly, 1×10^5 of BMDMs were seeded into each well of a 96-well plate. The following day, the overnight medium was replaced with serum-free media for 12 hours. Cells were then stimulated with different concentrations of MCC950 (AG-CR1-3615, Adipogen) (0.001, 0.01, 0.1, 1 μM) for 24 h. 10 μl of alamarBlue® reagent was added directly to cells in each well and incubated for 4 h at 37 °C. Then, absorbance at 570 nm was measured. Experiments were repeated three times.

Inflammasome activation assays

We seeded BMDMs, IP and MLN macrophages at 5×10^5/ml and LPMCs at $1x10^5$/ml in 96-well plates. The following day, the overnight medium was replaced, and cells were stimulated with 10 ng/ml LPS from *Escherichia coli* serotype EH100 (ra) TLRgrade (ALX-581-010-L001, Enzo Life Sciences) for 3 hours. The medium was removed and replaced with SFM containing MCC950 (AG-CR1-3615, Adipogen) (0.01 μM), glyburide (10238-21-8, Sigma-Aldrich) (200 μM), Cells were then stimulated with the following inflammasome activators: 5 mM adenosine 5′-triphosphate disodium salt hydrate (ATP) (A2382, Sigma-Aldrich) (1 hour), and 10 μM Nigericin (tlrl-nig, Invivogen) (1 hour). Supernatants were removed and analysed using ELISA kits according to the manufacturer's instructions IL-1β (DuoSet, R&D Systems), TNF-α (KMC3011, Invitrogen).

Western blotting

Cell lysates were prepared by direct lysis in 50 µl of 2× Laemmli sample buffer (1610737, Biorad). The protein content of supernatants was concentrated using StrataClean resin (400714, Agilent Technologies) according to the manufacturer's instructions. The protein samples were resolved on 4-20% mini-protean precast SDS-PAGE gels (4561093, Biorad) and transferred onto polyvinylidene difluoride membrane (1620177, Biorad) using transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3) in a wet-transfer system. Membranes were blocked in 5% (wt/vol) dried milk in TBS-T (50 mM Tris/HCL, pH 7.6, 150 mM NaCl and 0.1% (vol/vol) Tween-20) for 1 hour at room temperature. Membranes were incubated with primary antibody diluted in 5% (wt/vol) dried milk in TBS-T and then with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (sc-2030, Santa Cruz Biotechnology) diluted in 5% (wt/vol) dried milk in TBS-T for 1 hour. Membranes were developed using SuperSignal West Pico chemiluminescent substrate (34580, Thermo Fisher Scientific). Membranes were stripped using Restore PLUS Western blot stripping buffer (46430, Thermo Fisher Scientific) before being re-probed.

Primary antibodies used were ASC antibody (1 in 1,000) (AL177, Enzo Life Sciences); β-actin; mouse caspase-1 p10 (sc-514, Santa Cruz Biotechnology) (1:1,000), mouse IL-1β (NB600-633, Novus Biologicals) (1:1,000) and NLRP3 antibody (1:1,000) (NBP2-12446SS, Novus Biologicals). Secondary HRP-conjugated antibodies used were, anti-rabbit IgG and (1:5000) (sc-2030, Santa Cruz Biotechnology).

Densitometry analysis of Western blot images were performed in Multi Gauge analysis software Ver2.0 (FujiFilm).

***In vivo* oral administration of MCC950**

Seven-week-old Winnie mice (homozygous *Muc2* mutant; C57BL6/J background) n=20 of both sexes average weight 18 g were obtained from the University of Tasmania animal breeding facility. Mice were randomly divided into two groups. MCC950 used in *in vivo* experiment was a gift from Avril Robertson. MCC950 treatment group: (n=10) and control group (n=10). Treatment group mice were fed 1 g of freshly made chow mash (chow powder blended with water) mixed with 40 mg/kg MCC950 daily. Control mice received the formulation vehicle PBS in 1 g chow mash. The mice were single-caged throughout the

experiment to ascertain the defined daily intake of MCC950 from prepared chow mash. Mice were sacrificed on day 21.

Clinical scoring and histological analysis

Bodyweight, stool consistency and the presence of gross blood in stool and at the anus were observed every day. Stool was collected from individual mice and tested for the presence of blood using Hemoccult II slides (Beckman Coulter Inc., California, USA). The disease activity index (DAI) was calculated by assigning well-established and validated scores (Lean et al., 2015). Briefly, the following parameters were used for calculation: a) Stool consistency (0 points = normal, 1points = soft but formed, 2 points =loose stool, 3 points= watery stool; b) blood in stool (0 points=no bleeding, 1 point= Hemoccult +, 2 points= visual blood, 3 points=gross bleeding). At day 21 following treatment, animals were sacrificed by CO_2 asphyxiation. The colon from the caecum to the anus was removed. The length of the colon from ileocaecal junction to the rectum was recorded. The colon was subsequently opened along its longitudinal axis and the luminal contents were removed prior to weighing the organ. The colon was bisected longitudinally and one half was prepared using the Swiss roll technique (Cooper et al., 2000), whereas the remaining colonic tissue was dissected and snap-frozen for molecular analyses. Swiss rolls underwent 24 h fixation in 10% (v/v) neutral-buffered formalin (HT501128, Sigma-Aldrich). Swiss rolls were subsequently transferred to 70% ethanol prior to progressive dehydration, clearing and infiltration with HistoPrep paraffin wax (SH75-1250, Fisher Scientific). They were then embedded in wax and 5 μm sections were cut using a rotary microtome. Sections were stained with haematoxylin (HHS16, Sigma-Aldrich) and eosin Y (HT110280, Sigma-Aldrich). Slides stained with H&E were evaluated for inflammatory features. Histological inflammation was graded in a blinded fashion by RF and APP based on criteria (Randall-Demllo et al., 2016). Briefly, frequency of lamina propria neutrophils graded 0-2, frequency of crypt abscesses graded 0-2, crypt architectural distortion was graded 0-2, extent of surface damage graded 0-2, goblet cell depletion graded 0-2. The inflammation score for each individual region (distal, middle and proximal colon) was derived from the sum of the score for each of the aforementioned criteria.

Cytokine measurements

Serum was collected from blood drawn by cardiac puncture at the end of the treatment. Explants from the proximal and distal colons of treatment and control groups (n = 3) were

cultured overnight in RPMI 1640 (11875093, Sigma-Aldrich). Culture supernatants were measured for Nitrite by the Griess reaction method (G4410, Sigma-Aldrich) as an index of Nitric Oxide generation.

Cytokine concentrations in neat culture supernatants and serum were determined using mouse Bio-Plex mouse cytokine 23-plex panel kit (#M60009RDPD, Bio-Rad) and analysed using Bio-Plex 200 system (171000205, Bio-Rad) and Bio-Plex Manager software. IL-18 was determined by 5 times diluted supernatant measured by a mouse IL-18 ELISA kit (7625, R&D Systems). The most significantly altered cytokines are presented as pg per g of tissue.

RNA extraction and RT-PCR

Colonic tissue was homogenised using Omni Mixer Homogenizer (3410B05, Thomas Scientific) and RNA extracted using the RNeasy Mini spin column kit (74104, Qiagen) according to the manufacturer's instructions. Integrity and concentration of extracted RNA was assessed using Eppendorf Biophotometer (D30, Eppendorf). Complementary DNA (cDNA) was synthesised from RNA samples using the iScript gDNA clear cDNA synthesis kit (1725034, Bio-Rad) using reaction conditions suggested by the manufacturer. 100 ng of cDNA from each sample was added to a PCR reaction including TaqMan Fast Advanced Master Mix (4444557, Applied Biosystems) and a single gene-specific TaqMan probe/primer set. IL-1β (Assay ID: Mm00434228_m1, Thermo Fisher Scientific) and IL-18 (Assay ID: Mm00434226_m1, Thermo Fisher Scientific).

Thermal cycling was performed using a StepOnePlus Real-Time PCR Systems (4376600, Applied Biosystems). Gene expression was quantified using the comparative (ΔΔCT) method where the threshold cycle (CT) for each gene was normalised to reference gene Gapdh (Assay ID: Mm99999915_g1, Thermo Fisher Scientific). Relative gene expression in the MCC950 treated animals was presented as $2^{-\Delta CT}$.

NEK7 phosphorylation state analysis by Phos-tag SDS-PAGE

We seeded J774A.1 (TIB-67, ATCC) at 1×10^5/ml in 6-well plates. The following day, the overnight medium was replaced, and cells were stimulated with 100 ng/ml LPS (ALX-581-010-L001, Enzo Life Sciences) for 3 hours. The medium was removed and replaced with SFM containing MCC950 (AG-CR1-3615, Adipogen) (1 μM) and glyburide (10238-21-8, Sigma-Aldrich) (200 μM) for 30 min or no treatment with SFM. Cells were then

stimulated with 5 mM ATP (A2382, Sigma-Aldrich) for 30 min. Supernatants were removed and analysed using ELISA kits according to the manufacturer's instructions IL-1β (DY401, DuoSet, R&D Systems). Cell lysates from three wells were pooled in 200 ul of RIPA buffer (R0278 Sigma-Aldrich) with protease inhibitor (4693116001, Sigma-Aldrich). Contaminants were removed in protein samples by TCA precipitation. Samples mixed in 2× Laemmli sample buffer (1610737, Biorad) and run in Phos-tag SDS-PAGE (192-17401, Wako Chemicals) for the separation of phosphorylated proteins according to their degree of phosphorylation. Membranes were developed using SuperSignal West Pico chemiluminescent substrate (34580, Thermo Fisher Scientific). Membranes were stripped using Restore PLUS Western blot stripping buffer (46430, Thermo Fisher Scientific) before being re-probed. Primary antibodies used were NEK7 antibody (Ab109433, Abcam) (1 in 1,000). Secondary HRP-conjugated antibodies used were, anti-rabbit IgG and (1:5000) (sc-2030, Santa Cruz Biological).

Statistical analysis

Data are presented as average values ± SEM from multiple individual experiments each carried out in triplicate measurements in a representative experiment. Change in body weight percentage over time was compared using repeated-measures analysis of variance (ANOVA). The statistical significance of the normalised mRNA expression was tested by one sample t-test. Differences in histological scores between anatomical regions were tested post-ANOVA using Tukey's multiple pairwise comparisons test. Statistical analyses were done using a nonparametric an unpaired two-tailed *t*-test, for two groups study. The data were evaluated with one-way analysis of variance (ANOVA) for 3 groups study and confirmed using Tukey's test for multiple comparisons using Prism software (GraphPad). Data were considered significant when $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) or $P \leq 0.0001$ (****).

4.4 Results

4.4.1 MCC950 inhibits the activation of NLRP3 inflammasome in mouse macrophages

IL-1β is processed from the inactive cytoplasmic precursor pro-IL-1β which has to be cleaved by caspase-1 to produce the mature active form. We examined the ability of MCC950 to inhibit the activation of pro-IL-1β by inhibiting the activation of NLRP3 inflammasome. For our initial experiment we used a concentration of 0.01 µM of MCC950

which is close to 0.0075 µM, the half-maximal inhibitory concentration (IC_{50}) of MCC950 in bone marrow derived macrophages (BMDM) of C57BL/6 mice. We isolated BMDM, Intraperitoneal (IP), mesenteric lymph node (MLN) and lamina propria mononuclear cell (LPMC) murine macrophages from Winnie and C57BL/6 mice. We could not isolate enough MLN and LPMC from C57BL/6 for inflammasome activation experiments.

The results showed IL-1β release was markedly increased in the macrophages of Winnie mice compared with C57BL/6 upon LPS treatment. Cells were then pre-treated with MCC950 or glyburide and then stimulated with the NLRP3 agonists ATP or the ionophore nigericin. Treating cells with 0.01 µM of MCC950 and 200 µM glyburide significantly inhibited the release of IL-1β in BMDMs (Figure 4.1a), IPs (Figure 4.1b) and MLNs (Figure 4.1c). Complete inhibition of IL-1β was observed in LPMCs treated with MCC950 at 1 µM and stimulated with specific NLRP3 stimulants ATP and Nigericin (Figure 4.1d). LPS-dependent TNF-α secretion was not impaired by MCC950 in BMDMs (Figure 4.1e), which demonstrates that the inhibition of IL-1β secretion was specific. To investigate the potential cytotoxicity effect of MCC950, we performed the alamarBlue® cell viability assay. The results show that the there is no cytotoxic effects on Winnie BMDM cells against MCC950 at 0.001 µM-1 µM (Figure S1).

The amount of active caspase-1 p10 was reduced in supernatants from MCC950-treated Winnie and Wild type BMDMs (Figure 4.1f), suggesting that MCC950 inhibits the activation of caspase-1 by NLRP3. Correspondingly, the processing of IL-1β was inhibited by MCC950. Similarly, treatment with glyburide inhibited caspase-1 activation and IL-1β processing. Western blot densitometry analysis revealed that expression of the inflammasome complex proteins such as NLRP3 and ASC was not significantly changed during treatment with MCC950 (Figure S2(f)).

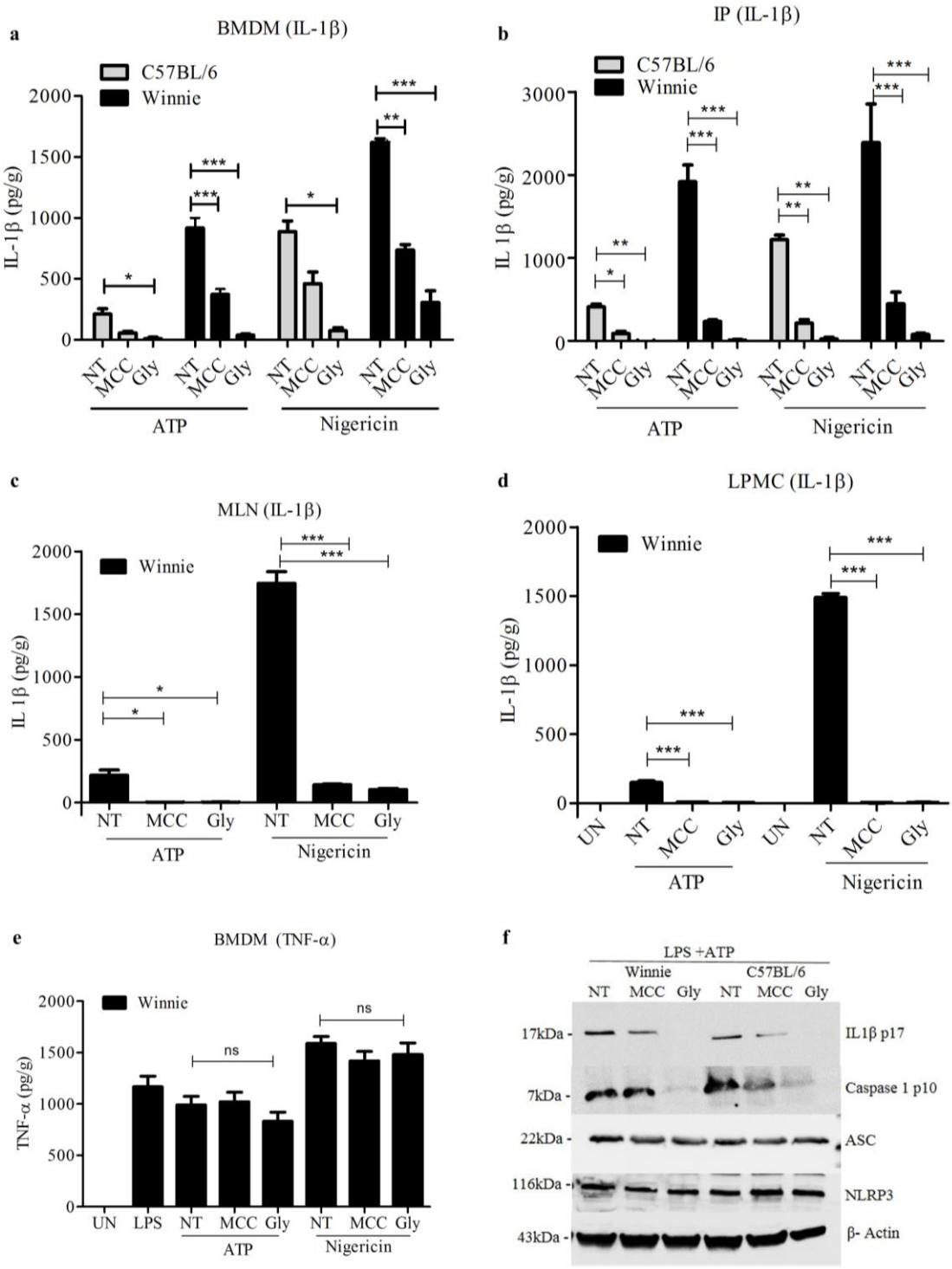


Figure 4.1: The effect of MCC950 on NLRP3 inflammasome activation in murine macrophages. Production of IL-1β (a) C57BL/6 and Winnie BMDMs (b) C57BL/6 and Winnie IP Macrophages (c) Winnie MLNs (d) Winnie LPMCs. Unprimed (UN), primed with 10 ng/ml LPS and treated with MCC950 (MCC) (a-c-0.01 µM, d-1 µM) and Glyburide (Gly) (200 µM) and stimulated with ATP and Nigericin as measured by ELISA. (e) Production of TNF-α for Winnie BMDM supernatants treated with MCC950 0.01 µM and glyburide 200 µM and stimulated with ATP and Nigericin as measured by ELISA. Data are expressed as the mean ± sem of three independent experiments carried out in duplicates. *P < 0.05, **P < 0.01, ***P < 0.001 (one-way ANOVA with Tukey's post-hoc test). (f) Western blots of cell lysates and supernatants from C57BL/6 and Winnie BMDMs

primed with 10ng/ml LPS and treated with MCC950 (0.01 μM) or glyburide (200 μM) and stimulated with ATP. These results are representative of three independent experiments.

4.4.2 MCC950 inhibits the activation of NLRP3 inflammasome in colonic explants

To further explore the effect of MCC950 on NLRP3 inflammasome activation in colitis, we investigated the release of IL-1β in treated Winnie colonic tissue explants by ELISA. Non-treated Winnie distal colon produces 12, 307 pg/g which is comparatively higher than the 7623.5 pg/g released by proximal colon tissue. MCC950 exhibited a concentration-dependent inhibition of IL-1β secretion from LPS treated Winnie proximal and distal colon explant tissue (Figure 4.2a). Treating colon explant with 10μM concentration of MCC950 was able to significantly reduce the release of IL-1β in proximal colon to 48.6% $P<0.001$ and the distal colon to 56.2% $P< 0.01$ (Figure 4.2b). LPS-dependent tumour necrosis factor-α (TNF-α) secretion was not impaired by 1 μM MCC950 and 200 μM glyburide (Figure 4. 2c) which demonstrates that the inhibition of IL-1β secretion was specific. The Western blot analysis showed that 1 μM MCC950 and 200 μM glyburide significantly inhibited the activation of caspase-1 in to the cleaved form caspase-1 p10. Correspondingly, 1 μM MCC950 and 200 μM glyburide suppressed the processing of proIL-1β to mature IL-1β (Figure 4.2d).

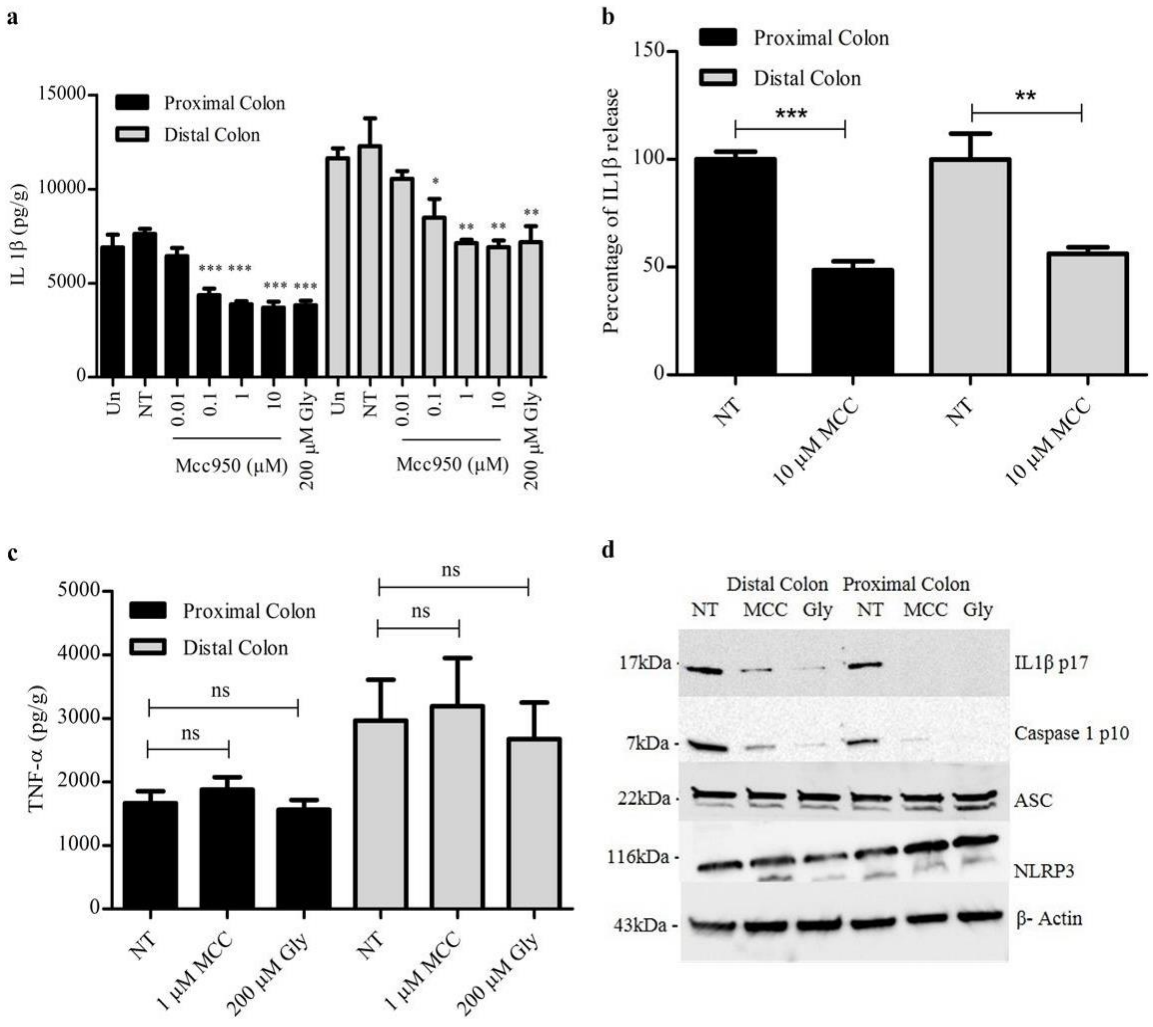


Figure 4.2: MCC950 inhibits NLRP3 inflammasome activation in colonic explants. (a) Production of IL-1β from Winnie proximal and distal colons stimulated with 10ng/ml LPS and treated with MCC950 (MCC) (0.001-10 µM) and Glyburide (Gly) 200 µM as measured by ELISA. (b)Percentage of IL-1β release of Winnie proximal and distal colons stimulated with LPS no treatment compared to treated with MCC950 (10 µM) as measured by ELISA. (c) Production of TNF-α for proximal and distal colonic explant supernatants treated with MCC950 (1 µM) and glyburide 200 µM as measured by ELISA. Data are expressed as the mean±SEM of five independent experiments carried out in duplicates. *P < 0.05, **P < 0.01, ***P < 0.001 (one-way ANOVA with Tukey's post-hoc test). (d) Western blots of tissue lysates and Supernatants from proximal and distal colons stimulated with 10ng/ml LPS and treated with MCC950 (1 µM) or glyburide (200 µM). These results are representative of three independent experiments.

4.4.3 Oral administration of MCC950 attenuates colonic inflammation in Winnie

The macrophage cell *in vitro* data and colon explant data suggested that MCC950 effectively inhibited NLRP3 inflammasome assembly and may be key to controlling colitis. To assess this, we examined the effect of MCC950 (40 mg/kg) in seven-week-old Winnie mice, administered orally for three weeks, in a spontaneous colitis mouse model Winnie. Throughout the experiment, mice were monitored for the clinical symptoms of colitis. Macroscopic observation of the 10-week control Winnie colon at experimental termination showed the colons to be visibly inflamed with shortening and thickening of colon wall with enlarged mesenteric lymph nodes when compared to MCC950 treated 10-week Winnie in (Figure 4.3a). The mean colon length of the MCC950 treated group 8.390±0.1080 was significantly (P<0.01) longer than the mean colon length of the control group 7.870 ± 0.01212 (Figure 4.3b).

MCC950 40 mg/kg prevented the shortening of the colon which is positively related to the severity of colitis. Wet colon weight, an indicator of intestinal oedema and inflammation, was presented as the ratio of colon weight over body weight (g/g). The untreated colitis group showed the highest relative weight 0.02152±0.00096. MCC950 treatment significantly (P<0.05) reduced the mean to 0.01902±0.00060 (Figure 4.3C).

To determine the therapeutic potential of MCC950 on colitis we characterised the control and treatment group by clinical parameters such as percentage of body weight gain and disease activity index (DAI) which is an average score of stool consistency, and blood in stool. As shown in Figure 4.3d in contrast to the control mice which gained 10.59% body weight over 21 days, MCC950 treated mice showed an average of 15.07% body weight increase. However, the body weight increase was not statistically significant between the control and treatment groups. MCC950 40 mg/kg significantly improved the DAI as early as the 9th day of treatment showing the highest significance at day 21 P<0.001 (Figure 4.4a). The increase in body weight correlated with a significant decrease in DAI for the MCC950 treated group. The relationship between these two clinical parameters was significant at P<0.0001 with a spearman's correlation of r=0.9342.

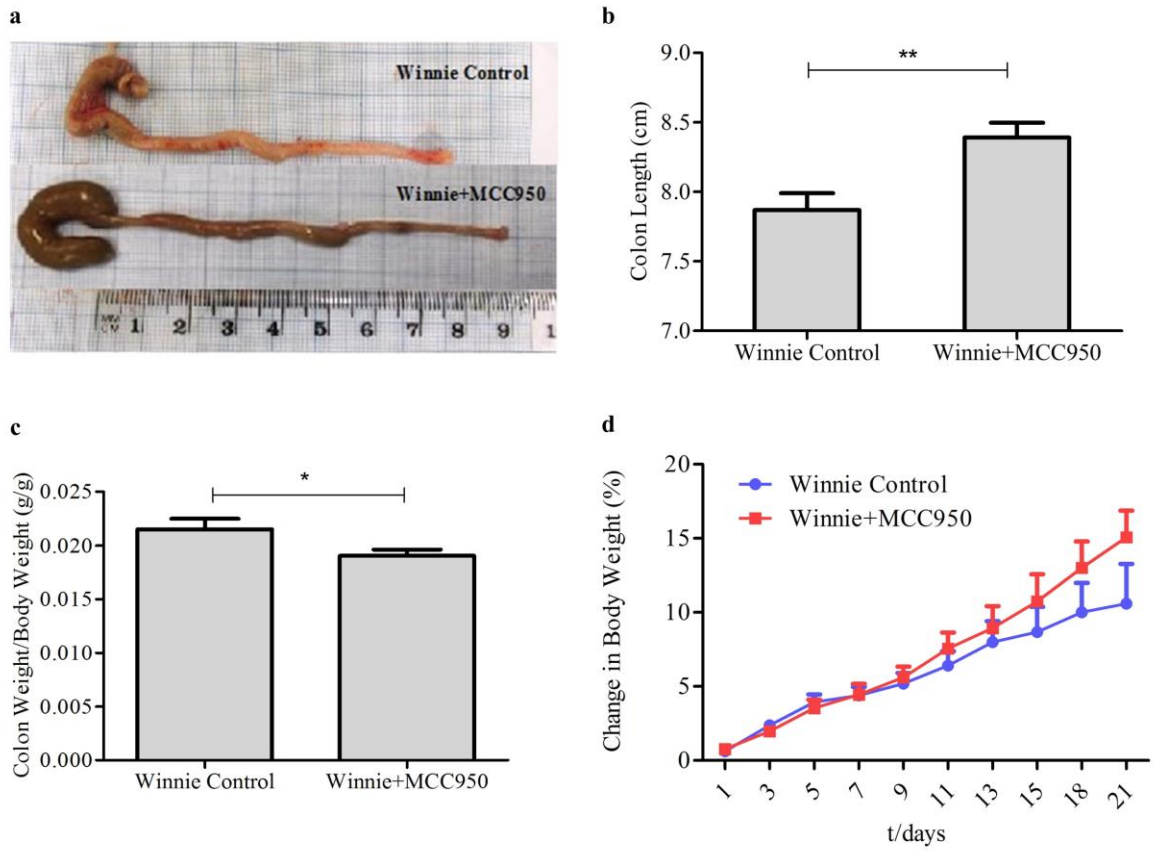


Figure 4.3: Effect of MCC950 on Winnie. Winnie at 10 weeks were weighed on the day of termination. Lengths of the freshly removed colons from each group were measured from ileocecal junction to rectum. The weight of the colons after removing luminal content was recorded. (a) Macroscopic appearances and (b) Colon Length for each group. (c) Ratio of colon weight over body weight. Data are expressed as the mean±SEM (n=10 per group) *P < 0.05, **P < 0.01 (two-tailed Student's t test). (d) Body weight of mice was measured every 3 days and presented as a percentage of their initial weight. Data are represented as means ± SEM (n=10 per group) repeated-measures analysis of variance (ANOVA).

Histological analysis showed infiltration of neutrophils (Figure 4.4c), severe surface epithelial damage (Figure 4.4d), crypt abscesses (Figure 4.4e), distortion of crypt architecture (Figure 4.4e), and complete loss of crypts (Figure 4.4e), particularly in the distal colon of colitis mice compared to MCC950 treated mice (Figure 4.4f-h). The results of standard pathological examination of mouse colon reflected in a histological score showed much improvement in pathological changes in mice treated with 40 mg/kg of MCC950 which was statistically significant for distal colon at a $P<0.001$. The comparative histological score of the mid colon was lower in the MCC950 group, however it was not statistically significant. These data reveal that MCC950 improves clinical and histological changes in the colon associated with spontaneous colitis.

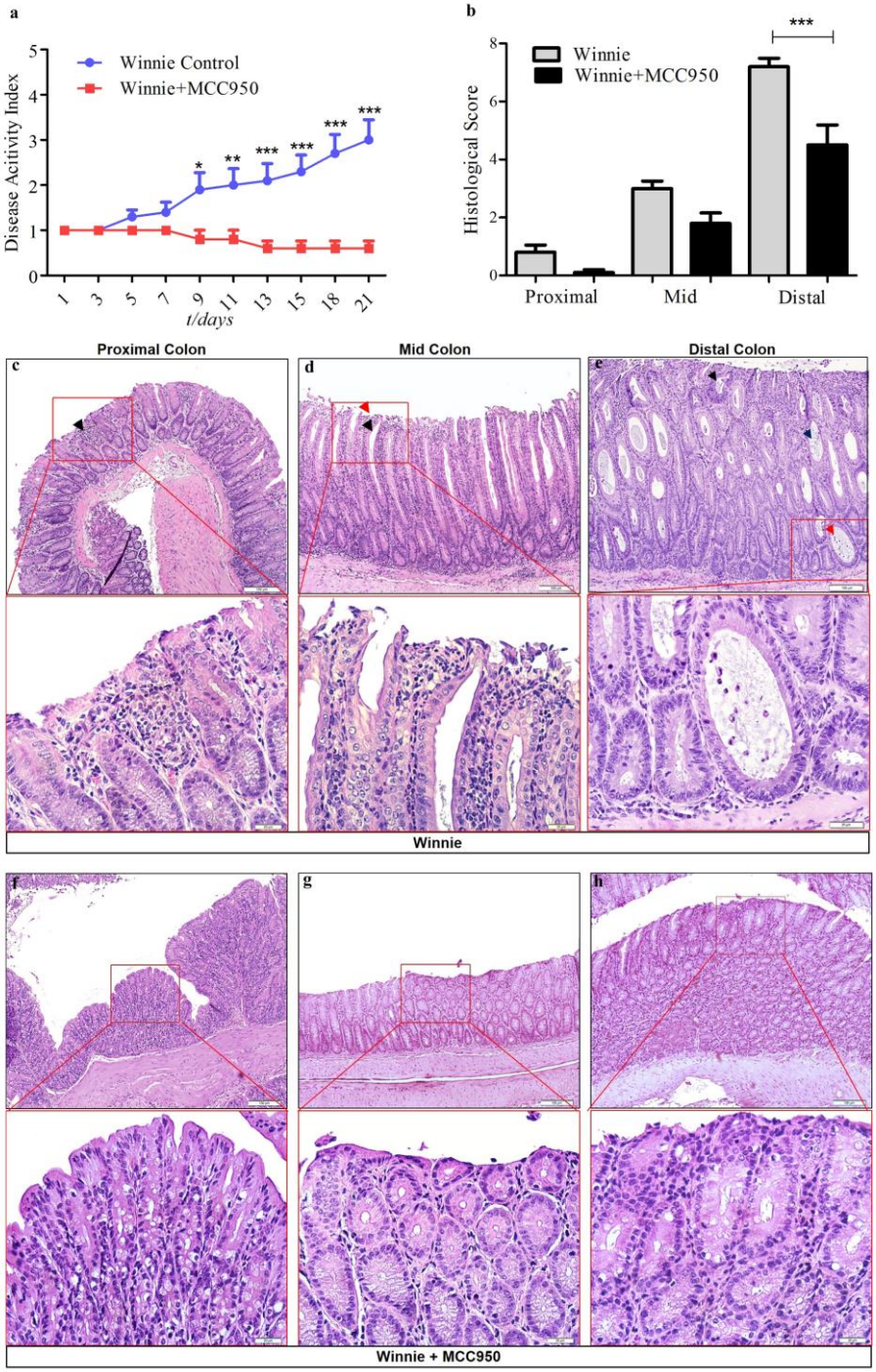


Figure 4.4: MCC950 treatment improves colitis in 10-week-old Winnie. (a) Disease activity index. (b) Comparison of summed inflammation scores between control and treatment Winnie mice. PC, proximal colon, MC, middle colon, DC, Distal Colon. Data are represented as means ± SEM (n=10 per group) *P < 0.05, **P < 0.01, ***P < 0.001 (one-way ANOVA with Tukey's post-hoc test). (c-e) Representative Winnie control proximal, middle and distal colon sections stained with hematoxylin and eosin at 100x and 400x. (c) Lamina propria inflammatory cell infiltrates (black arrow) (d) epithelial surface damage (red arrow), goblet cell loss (black arrow) (e) Crypt abscesses with neutrophils in the lumen and nearly intact epithelium (red arrow) or damaged epithelium and complete crypt loss (blue arrow) and crypt architectural distortion (black arrow). (f-h) Representative MCC950 treated Winnie proximal, middle and distal colon sections stained with hematoxylin and eosin at 100x and 400x.

4.4.4 Oral administration of MCC950 suppresses colonic IL-1β and IL-18 expression in Winnie

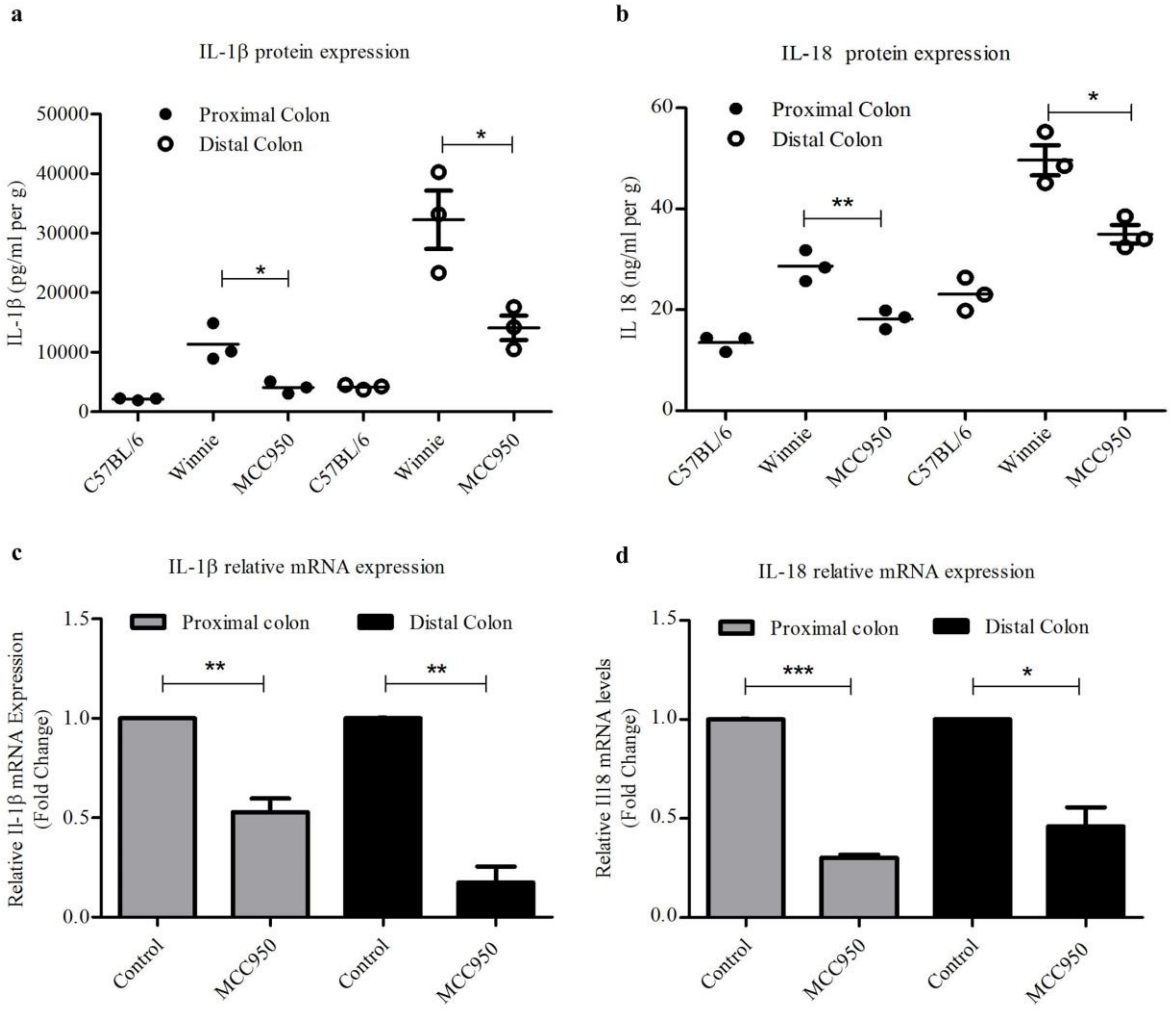


Figure 4.5: MCC950 suppressed NLRP3 activated proinflammatory cytokine levels in colon explant of Winnie mice. Protein levels of cytokines (a) IL-1β (b) IL-18 in C57BL/6, Winnie and MCC950 treated Winnie proximal and distal colon explant supernatants as determined by Bio-plex. Data presented as means ± SEM (n=3 per group) *P < 0.05, **P < 0.01 (two-tailed Student's t test). The mean values of fold change in mRNA expression levels for (c) IL-1β (d) IL-18 in MCC950 treated Winnie proximal and distal colon tissue are shown relative to the untreated Winnie proximal and distal control samples respectively. Both control and treated values were normalised to those of the internal control *GAPDH*, with treated values representing the fold change relative to that of controls, which was converted to 1. Data are expressed as the mean±SEM (n=4 per group) *P < 0.05, **P < 0.01 ***P < 0.001 (one sample t test).

To determine the effect of MCC950 on IL-1β and IL-18 cytokine production in colitis mice, cytokine expression in colonic tissue at both mRNA and protein levels in both groups were measured. MCC950 treatment was able to significantly (P<0.05) suppress IL-1β cytokine in proximal and distal colon compared to control group colons (Figure 4.5a). The suppression of Il-18 was at a significant level at proximal colon P<0.01 and distal colon at

P<0.05 (Figure 4.5b). Total RNA of colons was extracted and analysed for cytokine mRNA expression using quantitative real time PCR method. MCC950 treatment was able to significantly suppress IL-1β mRNA relative expression in proximal colon to 0.5277 (*P*<0.01) and distal colon to 0.1749 (*P*<0.01) (Figure 4.5c) and IL-18 mRNA relative expression in proximal colon to 0.3016 (*P*<0.001) and distal colon to 0.4606 (*P*<0.01) (Figure 4.5d).

4.4.5 Oral administration of MCC950 reduces colonic proinflammatory cytokines

While the mucosal explants isolated from Winnie colon control group actively secreted multiple proinflammatory cytokines (IL1-α, IFN-γ, TNF-α, IL-17 and IL-6), chemokine (MIP1a) and Nitric Oxide during 24-hour culture, MCC950 treatment effectively suppressed their release (Figure 4.6).

IL1-α was highly suppressed in MCC950 treated proximal and distal colons (*P*<0.01) (Figure 4.6a). IFN-γ was suppressed in MCC950 treated proximal colon, however it was not statistically significant. IFN-γ was strikingly suppressed in the distal colon (*P*<0.001) (Figure 4.6b). TNF-α was suppressed in MCC950 treated proximal colon however it was not statistically significant. In the distal colon TNF-α was significantly suppressed (*P*<0.001) (Figure 4.6c). MIP1a also known as the CCL3 chemokine was suppressed in MCC950 treated proximal colon however it was not statistically significant. In the distal colon MIP1a was effectively suppressed (*P*<0.01) (Figure 4.6d). IL-17 was highly suppressed in MCC950 treated proximal and distal colons (*P*<0.05) (Figure 4.6e). IL-6 was also highly suppressed in MCC950 treated proximal and distal colons (*P*<0.01) (Figure 4.6f). Interestingly MCC950 had no effect in blood plasma proinflammatory cytokines (IL1-β, IL1-α, TNF-α, IFN-γ, IL-17 and IL-6) and chemokine (MIP1-a) levels when compared to control group at the termination day at 24 hours after final treatment (Figure 4.6g). Nitrite was measured as an index of Nitric Oxide generation and was highly suppressed in MCC950 treated proximal and distal colons (*P*<0.01) (Figure 4.6h).

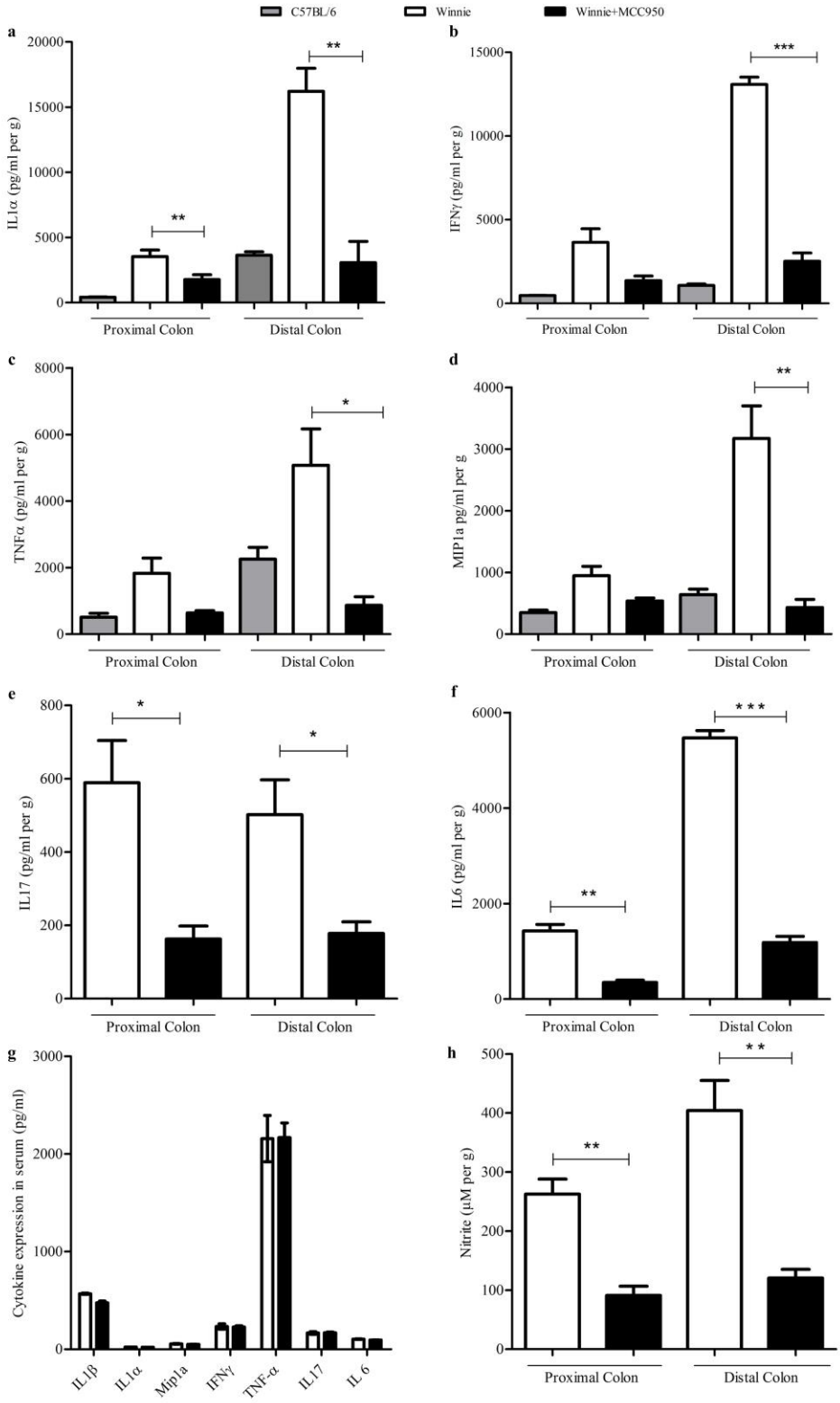


Figure 4.6: MCC950 suppressed proinflammatory cytokine and chemokine production in colon tissues but not in blood serum. Protein levels of cytokines including (a) IL1α (b) MIP1a (c) IL17 (d) IFN-γ (e) TNF-α in explant supernatants (f) IL-1β, IL-1α, MIP1a, IL17, IFN-γ, and TNF-α in blood serum were determined by Bio-plex. Data are presented as means±SEM (n=3) *P < 0.05, **P < 0.01, ***P < 0.001 (two-tailed Student's t test).

4.4.6 MCC950 and Glyburide do not target NEK7-NLRP3 interaction

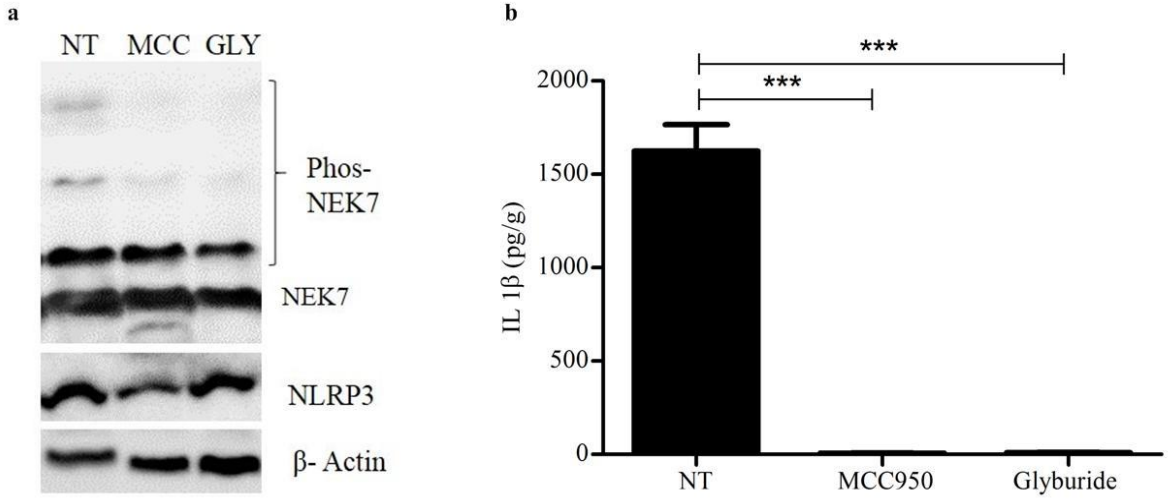


Figure 4.7: J774A.1 cells were primed with 100 ng/ml LPS and inhibited with 1 µM MCC950 or 200 µM Glyburide then stimulated with 5 mM ATP. (a)The phosphorylation state of NEK7 was analysed using Phos-tag SDS-PAGE. These results are representative of three independent experiments. (b) ELISA analysis of IL-1β in the culture supernatant of J774A.1 cells treated as in (a). Data are expressed as the mean ± sem of three independent experiments carried out in duplicates. ***P < 0.001 (one-way ANOVA with Tukey's post-hoc test).

NEK7 undergoes phosphorylation during the interaction with NLRP3 component and the level of phosphorylation correlates to the activation of the inflammasome (Shi et al., 2016). To examine the possibility of MCC950 and glyburide targeting the NEK7-NLRP3 interaction, we assessed the phosphorylation component of NEK7. We primed the murine macrophage cell line J744A.1 with LPS and applied the inhibitors and activated the NLRP3 inflammasome with ATP and analysed the level of NEK7 phosphorylation using Phos-tag SDS-PAGE. The Phos-tag SDS-PAGE blot analysis showed that the level of phosphorylated NEK7 band was the same size for MCC950 and glyburide and the untreated sample (Fig. 4.7a). This result concludes that both the inhibitors did not react by inhibiting the NEK7-NLRP3 interaction. The IL-1β levels were suppressed by the inhibitors, recognizing that the inhibitors were effective (Fig. 4.7b).

4.5 Discussion

The study describes for the first time the oral administration of MCC950 in the spontaneous colitis murine model Winnie. The most salient finding of the study is that MCC950 decreased the severity of chronic colitis in Winnie mice by specific inhibition of NLRP3 inflammasome activation. In addition, MCC950 treatment significantly decreased the expression of proinflammatory cytokines IL-1β and IL-18 at mRNA and protein level and the associated release of proinflammatory cytokines and chemokine in Winnie colonic

tissue. Moreover, MCC950 resulted in a significant decrease of IL-1β and active caspase-1 in Winnie explants and *in vitro* macrophage cells isolated from Winnie mice. Our data shows that the NLRP3 inflammasome plays a negative role in the chronic phase of ulcerative colitis in Winnie. The results collectively suggest that MCC950 acts as an effective therapeutic compound for the treatment of murine ulcerative colitis.

The results show that IL-1β is released more in the Winnie distal colon when compared to the proximal colon. This explains why MCC950 is more effective in the distal colon and shows more improvement with MCC950. We further analyzed the Winnie explant IL-1β ELISA data treated at 10 μM, as this high dose will ensure complete efficacy in NLRP3 inflammasome inhibition even in the presence of explant serum. The results show that the NLRP3 specific inhibitor MCC950 at 10 μM inhibited the release of IL-1β in proximal colon by 51.4% and the distal colon by 43.7 %. This is an important finding as this indicates the percentage of canonical and non-canonical NLRP3 inflammasome contribution to the overall IL-1β in the spontaneous colitis colon of Winnie mice. High NLRP3 expression has been found in the ulcerated colonic tissue and in the colon of mice with acute and chronic colitis (Villani et al., 2009). Accumulating evidence support a pro-inflammatory contribution of NLRP3 to colitis pathology (Allen et al., 2010, Liu et al., 2013). It is reported that blockage of IL-1β (Thomas et al., 1991) or neutralization of IL-18 (Sivakumar et al., 2002, Ten Hove et al., 2001) reduces intestinal inflammation. These findings support the detrimental role of the NLRP3 inflammasome in the development of spontaneous colitis in Winnie which is supported by both DSS model (Bauer et al., 2010) and *Il-10*$^{-/-}$ model of colitis (Zhang et al., 2014a). The explant results show that selective inhibition of the NLRP3-inflammasome with MCC950 dose dependently reduces activated IL-1β secretion from colon tissue simulating a clinically feasible treatment regimen.

A study by Bauer et al., 2010 has proved that the mechanism of NLRP3/ASC/caspase-1 mediated activation of proinflammatory IL1-β and IL-18 is essential for experimental colitis (Bauer et al., 2010). The Western blot results of the *in vitro* experiments showed that treatment with MCC950 significantly reduced the active caspase-1 p10 and IL-1β in BMDMs and colon explants suggesting that MCC950 inhibits the activation of NLRP3 inflammasome in the colitis model. Similarly, treatment with glyburide inhibited caspase-1 activation and IL-1β processing as seen in protein quantification assays.

For the *in vitro* experiments we chose to use primary macrophages because NLRP3 and ASC complex is mainly expressed in these innate immunity cells (Kummer et al., 2007) and they have a vigorous inflammasome activation and IL-1β production. The primary macrophage cells isolated from Winnie had a higher secretion of IL-1β in comparison to C57BL/6 indicating active colitis in Winnie. Similarly it is seen in murine DSS models (Arai et al., 1998) and human colitis patients (Ligumsky et al., 1990) where active colitis and its severity has been correlated to high levels of IL-1β secreted by activated macrophages. Intestinal inflammation is associated with infiltration of macrophages into the colon lamina propria where a variety of inflammatory cytokines are produced in response to intestinal microbes. A very recent research in gut microbiota has shown that NLRP3 is absent in the epithelium but present in the deeper residing lamina propria mononuclear cells (Yao et al., 2017a). The *in vitro* results on LPMCs show complete inhibition of IL-1β by 1 μM of MCC950, which explains how MCC950 attenuated colonic inflammation in the *in vivo* spontaneous colitis model Winnie.

For *in vitro* experiments apart from MCC950 we have also used glyburide, another specific inhibitor of NLRP3 inflammasome (Lamkanfi et al., 2009). A recent study investigated the effect of glyburide in *Il-10*$^{-/-}$ spontaneous Crohn's disease mouse model (Liu et al., 2016a). Very similar to the results with MCC950 in Winnie mice, glyburide effectively suppressed NLRP3 inflammasome activation in *Il-10*$^{-/-}$ mice, leading to attenuation and prevention of colitis. Moreover, glyburide also effectively inhibited the release of proinflammatory cytokines and chemokines in a similar manner to MCC950 effect in the Winnie model. Our *in vitro* results with glyburide show that it is effective in inhibiting NLRP3 inflammasome in a spontaneous ulcerative colitis mouse model. Further *in vivo* studies should be conducted to evaluate the potency of glyburide in the Winnie. However in comparison to glyburide, MCCC950 has superior pharmacological characteristics such as higher potency (7.5 nM) , oral bioavailability (68%), temporal application and no known side effects (Coll et al., 2015). Current experimental NLRP3 inhibitors of colitis are applied parenterally (Guo et al., 2014, He et al., 2016a), however in our study we have shown effective oral administration with MCC950 which is more clinically desirable than parenteral application.

In the study MCC950 treatment significantly improved clinical parameters of body weight gain, colon length, bloody stool and stool consistency. Similarly, histopathological findings

supported that MCC950 protected Winnie mice from surface epithelial erosion, inflammatory cell infiltration, loss of goblet cells and disruption of crypt architecture. These findings indicate oral administration of specific NLRP3 inhibitor MCC950 at 40mg/kg decreased the severity of spontaneous chronic colitis in Winnie mice.

Ulcerative colitis is characterized by a disturbed balance between regulatory and effector cells which mainly implicates effector T cells (Th1 and Th2), regulatory T cells (T_{regs}) and Th17 cells (Marquez-Flores et al., 2016). The intestinal inflammation is characterized by a Th1- and Th17-mediated responses with enhanced expression of TNF-α, IFN-γ, IL-1β, IL-12, IL-6, IL-10 and IL-17 (Kmiec et al., 2017). In this regard, Winnie 10-week colon explants showed prominently significant increase in IL-1β, IL1-α, IL-18, TNF-α, IFN-γ, and MIP1-a when compared to C57BL/6 (Figure 4.6a-f). This establishes the experimental model 10-week-old Winnie, as a clinically relevant model of chronic ulcerative colitis. Importantly the results demonstrated that MCC950 treatment significantly reduced IL-1β, IL-1α, IL-17, IL-6, IFN-γ, TNF-α and MIP1-a in the colitis colon (Figure 4.6a-f). Similarly treatment with MCC950 has been reported to have reduced proinflammatory cytokines and chemokines in other inflammatory diseases such as Influenza A virus infection (Tate et al., 2016), in renal inflammation (Krishnan et al., 2016) and a dermal inflammation model (Primiano et al., 2016). MIP1-a (CCL3), is a chemokine that attracts proinflammatory cytokine production and is particularly alleviated in Winnie distal colon. However, after oral treatment with MCC950 the MIP1-a levels were significantly lowered. Increased generation of Nitric Oxide leads to excessive production of reactive nitrogen species resulting in infiltration of inflammatory cells and intestinal damage (Sánchez-Fidalgo et al., 2010). MCC950 treatment significantly reduced nitrite which is an index for Nitric Oxide production significantly in Winnie proximal and distal colon.

From these results we can conclude that MCC950 inhibition of NLRP3 inflammasome has indirectly suppressed the activation of infiltrating macrophages by inhibiting the release of pro-inflammatory cytokines, chemokine immunomodulators and Nitric Oxide that contributed to the chronic inflammatory process in the Winnie colon. MCC950 is absorbed into the blood stream and is cleared within a window of time. Initially it will be efficacious in the reduction of IL-1β, however proinflammatory cytokines measured in blood plasma taken after 24 hours of treatment did not show any effect on systemic cytokines. This

suggest that MCC950 did not have a prolonged adverse systemic effect but its potency on reducing proinflammatory cytokines was significant at the colonic tissue.

We investigated the possibility of MCC950 and glyburide target to be the NEK7-NLRP3 interaction. Shi et al has shown that NEK7 phosphorylation enhances its binding to NLRP3 and promotes inflammasome activation (Shi et al., 2016). Our results showed no difference in the level of phosphorylated NEK7 between the untreated and MCC950 or glyburide treated samples. However, the IL-1β levels were suppressed by the inhibitors conceding that they successfully blocked the activation of NLRP3 activation. This result suggest that the inhibitory target is not NEK7-NLRP3 but is downstream of this interaction.

The role of NLRP3 inflammasome in colitis is controversial. Genetic ablation of genes of NLRP3 components are predisposed to colitis and colorectal cancer (Allen et al., 2010). However, hyper activation of NLRP3 inflammasome leads to colitis (Neudecker et al., 2017). This stresses the need for careful investigation of temporal therapeutic strategies in different disease phases in clinically relevant age appropriate experimental models. The findings from Tate et al on MCC950 treatment for influenza A virus shows that blockade of NLRP3 is detrimental at early stage of disease while protective at late stage of disease (Tate et al., 2016). Interestingly the results show that MCC950 is therapeutic in ulcerative colitis at chronic phase of the disease. Further studies are needed to look at emerging colitis to choose the optimal clinical treatment point of MCC950 for ulcerative colitis.

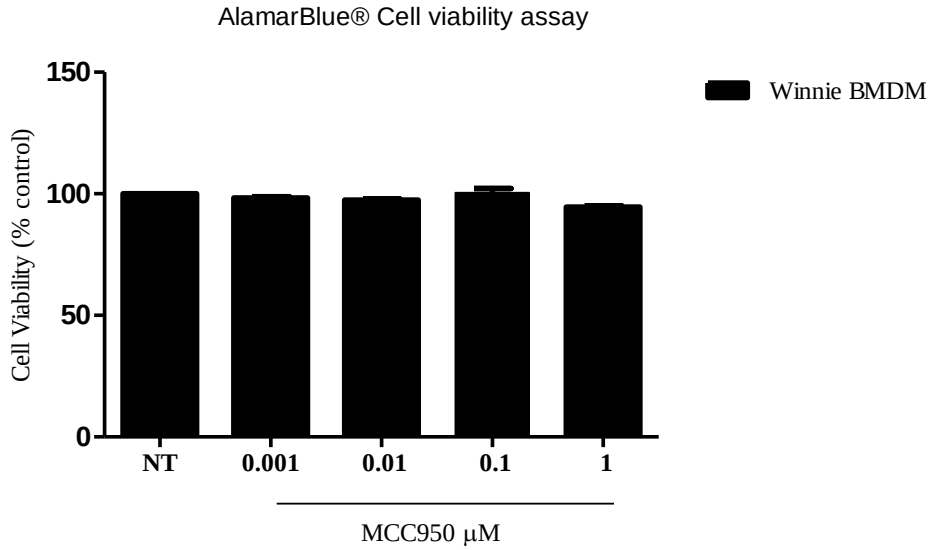
4.6 Conclusion

In conclusion, the results collectively suggest that the small molecule MCC950 paves the way for a novel therapeutic strategy in ulcerative colitis. The results show, for the first time, the contribution of anti-inflammatory effects resulting exclusively from inhibition of canonical and non-canonical NLRP3 inflammasome activation in colitis. Moreover, the ability of MCC950 to suppress both translational and transcriptional IL-1β and IL-18 of canonical and noncanonical NLRP3 inflammasome in the colon may be promising in inflammatory intestinal diseases other than ulcerative colitis. Nevertheless, the detailed mechanism of the pharmacological target of how MCC950 inhibits the activation of NLRP3 inflammasome needs to be explored in a future study.

4.7 Supplementary data

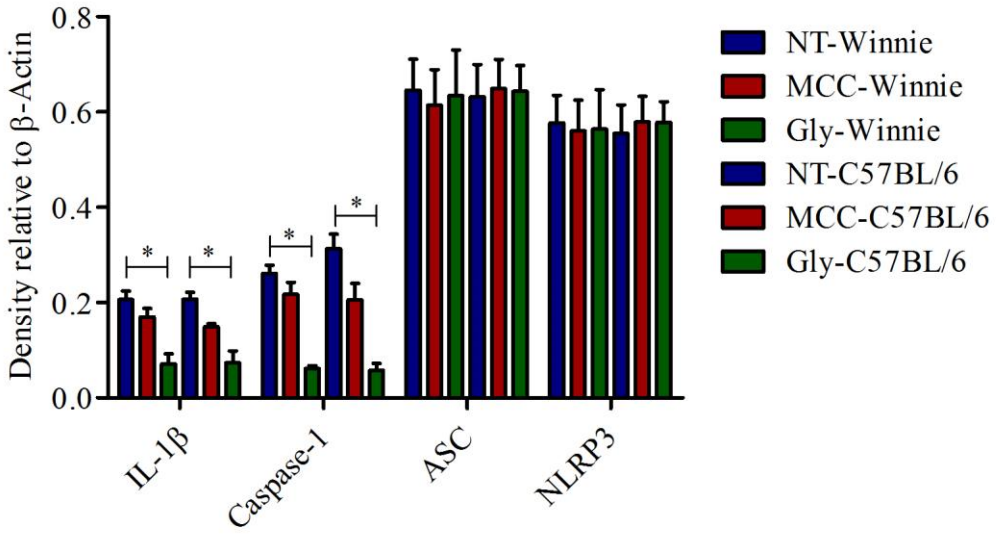
SUPPLEMENTARY FIGURE S1. AlamarBlue® Cell viability assay results.

Data represents the mean viability ± SEM from duplicate determinations and representative of three independent experiments.

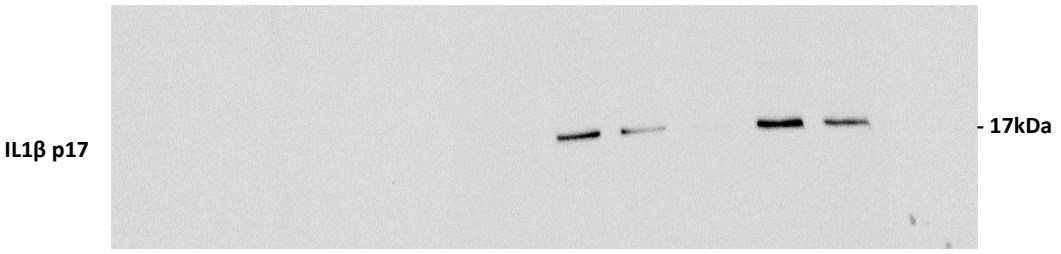


SUPPLEMENTARY FIGURE S2. Western blot densitometry analysis graph of IL-1β, caspase-1, ASC, NLRP3 density analysis relative to β-Actin The densitometry data are expressed as the mean ± SEM of n=3 experiments. *P < 0.05 (Two-way ANOVA with Bonferroni post-test). Uncropped Western blot images (b-f), represents full-length Western blot images of original Figure 4.1 (f).

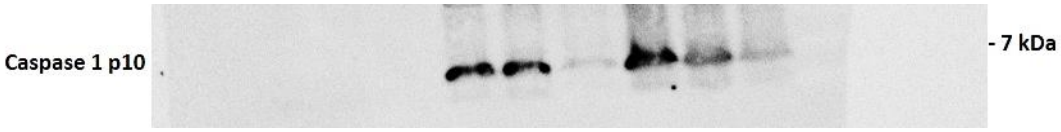
a



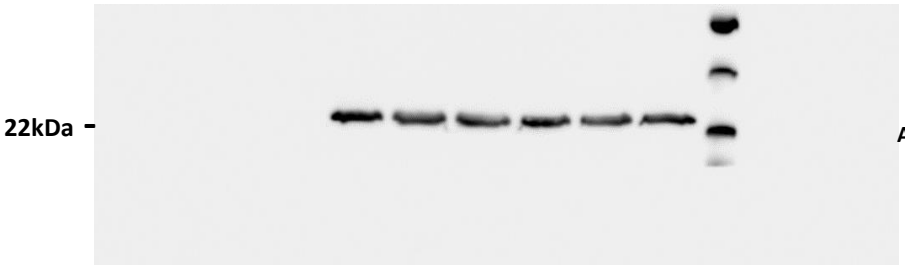
b


IL1β p17
- 17kDa

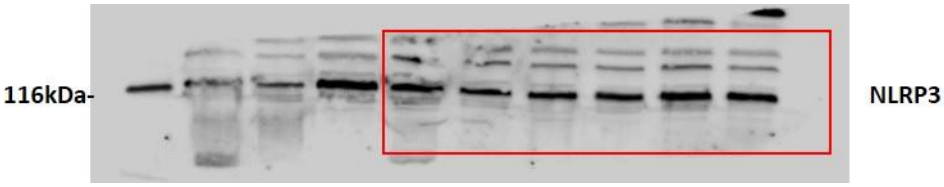
c


Caspase 1 p10
- 7 kDa

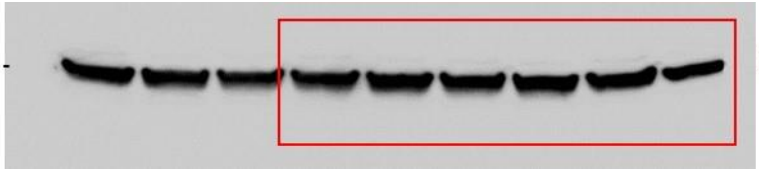
d


22kDa -
ASC

e

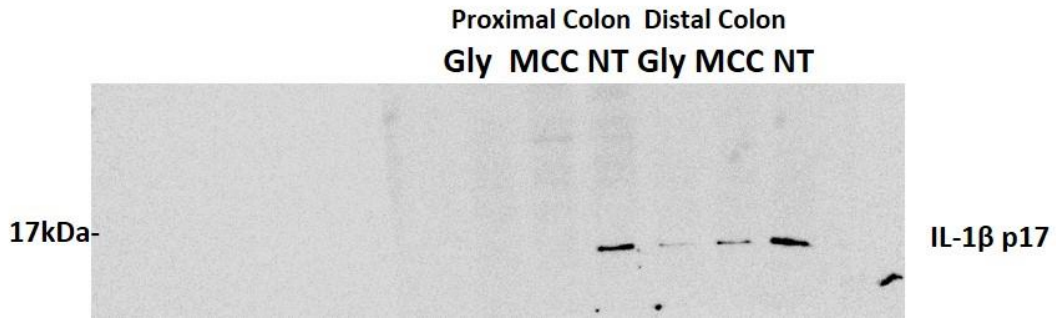

116kDa-
NLRP3

f

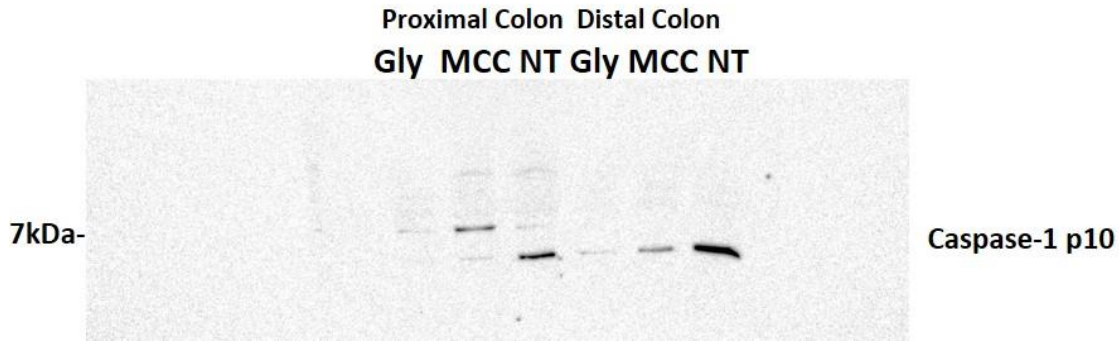

43kDa -
β- Actin

SUPPLEMENTARY FIGURE S3. Uncropped Western blot images (a-e). This figure represents full-length Western blot images of original Figure 4.2 (d)

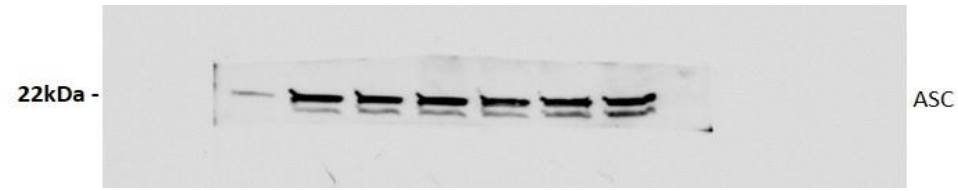
a



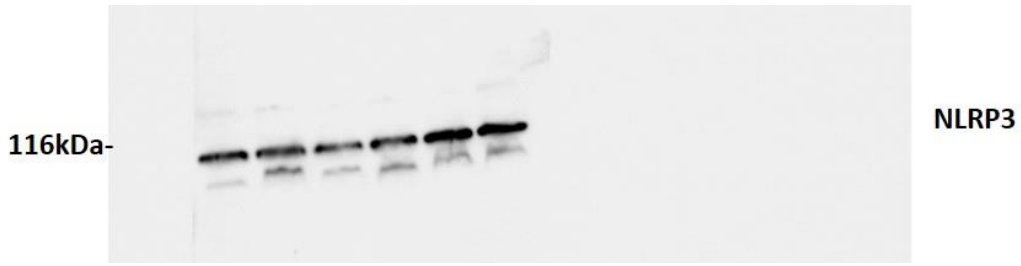
b



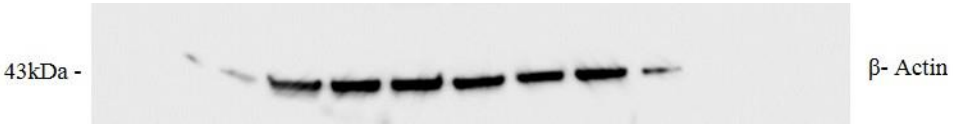
c



d

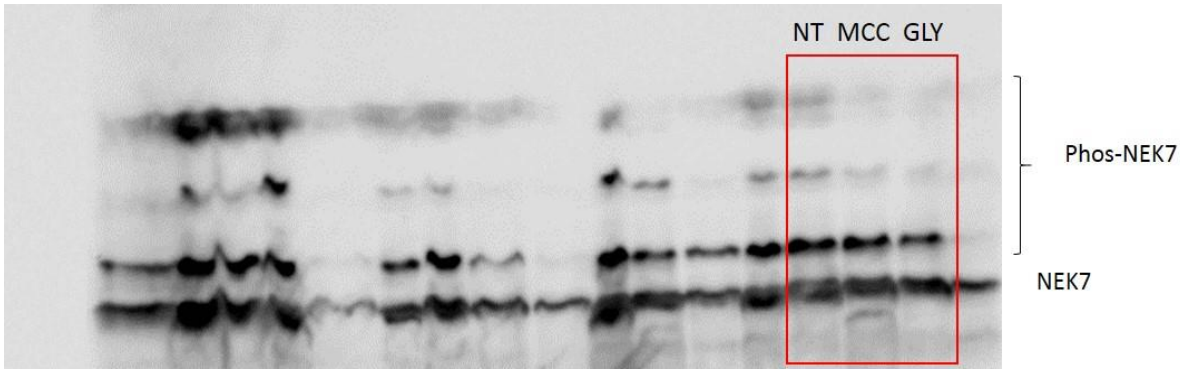


e

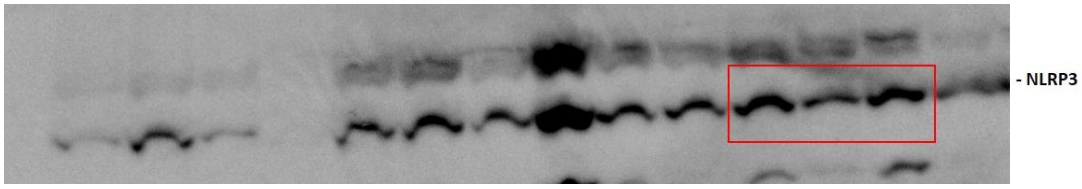


SUPPLEMENTARY FIGURE S4. Uncropped Western blot images (a-c). This figure represents full-length Western blot images of original Figure 4.7 (a).

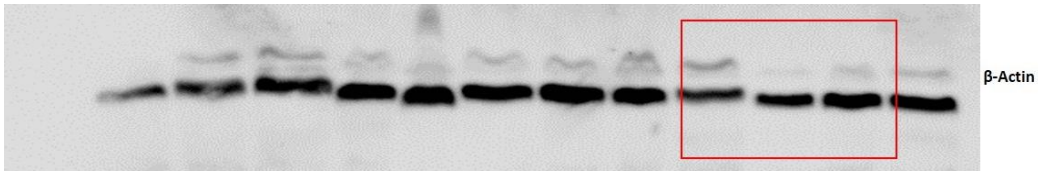
a



b



c



Chapter 5

Clinical, histopathological and cytokine assessment of Winnie x *Nlrp3*$^{-/-}$ mouse colon

5.1 Introduction

Nlrp3$^{-/-}$ mice has been used extensively to study the function of NLRP3 in the intestine through experimental colitis (DSS) and CAC (AOM/DSS) disease models. However, to date the results have been controversial with reports of both protective and deleterious effects (Zambetti and Mortellaro, 2014). The conflicting data in the above experimental models could be due to the different chemical methods used in colitis induction and alteration in the gut microbiota between different animal facilities. This study aimed to address the above experimental errors and examine intestinal immune pathways in carefully controlled spontaneous colitis mice deficient in the *Nlrp3* gene. The novel mouse model Winnie x *Nlrp3*$^{-/-}$ was generated to ascertain the functional role of NLRP3 inflammasome in spontaneous colitis. Please refer to section 2a.2 for detailed methodology of the generation of Winnie x *Nlrp3*$^{-/-}$.

Genetically modified *Nlrp3* mouse models are used for studying the role of the NLRP3 inflammasome in immune responses in many inflammatory diseases such as Muckle-Wells syndrome, colitis, metabolic syndrome, type 2 diabetes, non-alcoholic fatty liver disease, gout, atherosclerosis, multiple sclerosis, Alzheimer's disease and Parkinson's (Guo et al., 2015a).

Nlrp3^{R258W} mice carry a gain of function mutation in the gene coding for NLRP3 and were used to study diseases such as Muckle-Wells syndrome and familial cold autoinflammatory diseases caused by an excessive secretion of IL-1β (Hoffman et al., 2001, Martinon et al., 2009). However, *Nlrp3*^{R258W} mice with enhanced inflammasome signalling do not display an inflammatory pathology of the intestine. A recent study has shown that this is due to an excess of local IL-1β production, but not IL-18, that reshapes intestinal microbiota to induce local T_{regs}, to maintain intestinal homeostasis (Yao et al., 2017b).

Genetic *Nlrp3* deficiency in *Nlrp3*$^{-/-}$ mice attributes to a long-life span and has been shown to protect these mice from age related increases in inflammation (Youm et al., 2013). Another study comparing WT and *Nlrp3*$^{-/-}$ littermates discovered 10 bacterial genera

clearly enriched in the faecal microbiota of Nlrp3-deficient animals (Hirota et al., 2011). Additionally, we and others have found the colon phenotype of *Nlrp3*$^{-/-}$ mice to be healthy and similar to WT mice with base levels of inflammatory markers (Youm et al., 2013, Zambetti and Mortellaro, 2014).

The Winnie mice phenotype has been extensively studied based on clinical, histological, molecular and immunological aspects, and it is a well-established spontaneous chronic colitis model (Heazlewood et al., 2008, Eri et al., 2011, McGuckin et al., 2011). The involvement of both the adaptive and innate immune system is well showcased in Winnie mice colitis. Winnie mice develop spontaneous colitis without chemical induction and mimic human UC and hence the best option to explore the role of the NLRP3 function in colitis.

In this chapter, primary level assessment of Winnie x *Nlrp3*$^{-/-}$ mouse colon pertaining to clinical parameters, changes in the histopathology, biochemical analysis, and cytokine profile are outlined (Figure 5.1). The Winnie x *Nlrp3*$^{-/-}$ parameters were evaluated at age 12 weeks and 16 weeks in comparison to C57BL/6J (WT), Winnie and *Nlrp3*$^{-/-}$ control mouse models at 12 weeks. Both Winnie and *Nlrp3*$^{-/-}$ mouse models were generated from a C57BL/6J background.

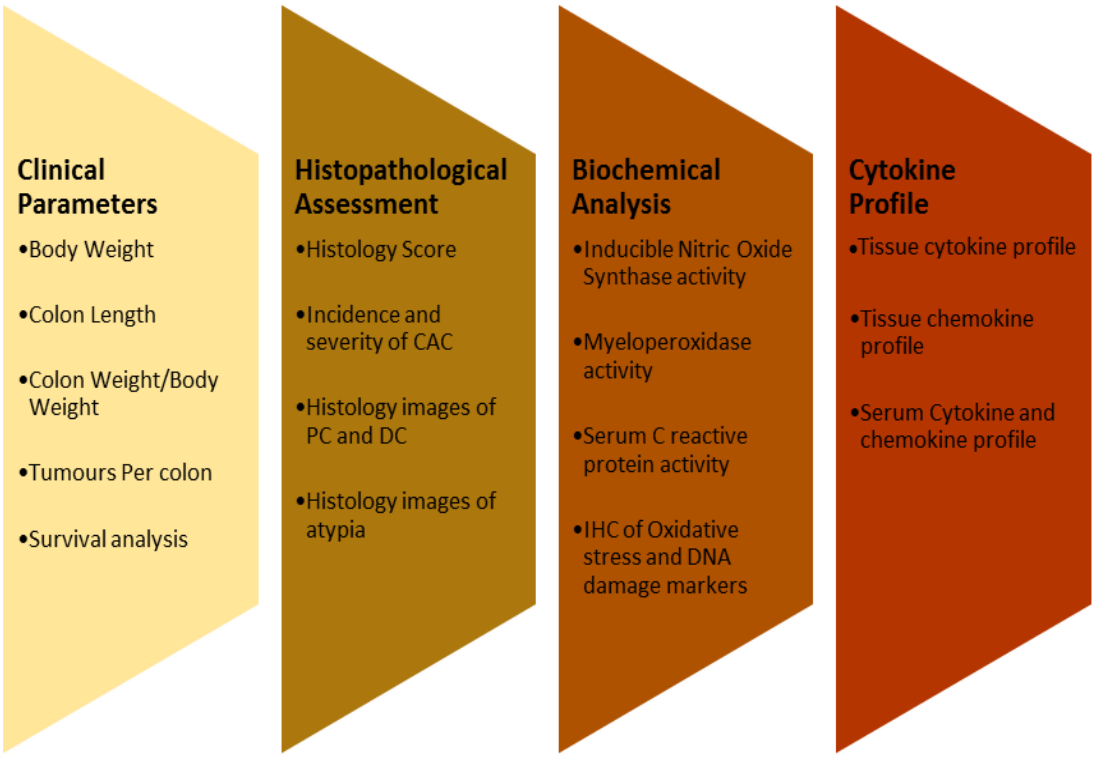


Figure 5.1: Schematic representation of phenotype analysis.

5.2 Methods

Animals

All animal experiments were approved by the Animal Ethics Committee of the University of Tasmania (Ethics approval number: A14095) and conducted in accordance with the Australian Code of Practice for Care and Use of Animals for Scientific Purposes (8th Edition 2013). Mice were housed in a temperature-controlled environment with a 12-hour day/night light cycle. Individual body weights were assessed daily over an initial acclimatisation period of 7 days. All mice had access to radiation-sterilised rodent feed (Barastoc Rat and Mouse, Ridley AgProducts, Australia) and autoclaved tap water for drinking ad libitum during experiments. All efforts were made to minimize animals' suffering and to reduce the number of animals used. Please refer to section 2a.2 for detailed methodology of the generation of Winnie x Nlrp3-/-.

Clinical analysis and histological scoring

At week 12 and week 16 animals of all four groups were sacrificed by CO2 asphyxiation. The colon from the caecum to the anus was removed. The length of the colon from ileo-caecal junction to the rectum was recorded. The colon was subsequently opened along its longitudinal axis and the luminal contents were removed prior to weighing the organ. The colon was bisected longitudinally, and one half was prepared using the Swiss roll technique, whereas the remaining colonic tissue was dissected and snap-frozen for molecular analyses. Swiss rolls underwent 24 h fixation in 10% (v/v) neutral-buffered formalin (HT501128, Sigma-Aldrich). Swiss rolls were subsequently transferred to 70% ethanol prior to progressive dehydration, clearing and infiltration with HistoPrep paraffin wax (SH75-1250, Fisher Scientific). They were then embedded in wax and 5 μm sections were cut using a rotary microtome. Sections were stained with haematoxylin (HHS16, Sigma-Aldrich) and eosin Y (HT110280, Sigma-Aldrich). Slides stained with H&E were evaluated for inflammatory features, hyperplasia, low and high-grade dysplasia and invasive carcinoma (Table 5.1). Histological scoring was performed in a blinded fashion by the Launceston General Senior Pathologist based on criteria detailed in Table 5.2. The histological score for each individual region (distal, middle and proximal colon) was derived from the sum of the score for each of the afore mentioned criteria.

Cytokine and Biochemical measurements

Serum was collected from blood drawn by cardiac puncture at the end of the treatment. Explants from the proximal and distal colons of treatment and control groups (n = 3) were cultured overnight in RPMI 1640 (11875093, Sigma-Aldrich). Cytokine concentrations in neat culture supernatants and serum were determined using mouse Bio-Plex mouse cytokine 23-plex panel kit (#M60009RDPD, Bio-Rad) and analysed using Bio-Plex 200 system (171000205, Bio-Rad) and Bio-Plex Manager software. IL-18 was determined by 5 times diluted supernatant measured by a mouse IL-18 ELISA kit (7625, R&D Systems). The most significantly altered cytokines are presented as pg per mg of tissue. Tissue homogenates were measured for myeloperoxidase activity using the colorimetric activity assay kit (ab105136, Abcam) according to manufacturer's instructions. Tissue homogenates were measured for inducible Nitric Oxide synthase activity using the inducible Nitric Oxide synthase fluorometric activity assay kit (ab211084, Abcam) according to manufacturer's instructions. Data presented as fold changes compared to WT group. Blood serum was measured for C reactive protein using the kit Mouse C-Reactive Protein/CRP Quantikine ELISA Kit (MCRP00, R&D Systems) according to manufacturer's instructions.

Immunohistochemistry

Slides were dewaxed and exposed to heat-induced epitope retrieval (4 min at 121 ℃) in a 10 mmol/L sodium citrate buffer (C9999, Sigma-Aldrich) pH 6.0 in a decloaking chamber (DC2012, Biocare Medical). Slides were cooled to room temperature and washed in 0.1 mol/L TBS (50 mM Tris/HCL, pH 7.6, 150 mM NaCl) for 2 min per wash. Endogenous peroxidase activity was blocked by incubating slides in 3% H_2O_2 (H1009, Sigma-Aldrich) in Methanol (322415, Sigma-Aldrich) for 20 min, followed by 3 × 2 min washes (twice with dH2O, followed by one wash with TBS). Background sniper (BS966, Biocare Medical) was applied to the slides for 20 min and washed off with 3 × 2 min washes with TBS. Slides were incubated with either anti-Oxoguanine 8 antibody (ab64548, Abcam), at a 1:300 dilution, Anti-gamma H2A.X (ab11174, Abcam) at a 1:300 dilution, Anti-NQO1 antibody (ab28947, Abcam) at a 1:300 dilution, Anti-3-Nitrotyrosine antibody (ab61392, Abcam) at a dilution of 1:300 was incubated with the slides for overnight. Excess primary antibody was removed with 3 × 2 min washes with TBS prior to application of HRP-conjugated anti-rabbit (M3R531, Biocare Medical) or anti-mouse secondary antibody

(MHRP520, Biocare Medical) for 30 min. Slides were thoroughly rinsed with TBS for 3 × 2 min washes before the addition of a diaminobenzidine (DAB) chromogen solution (DB801, Biocare Medical) for 4 min. Tissue was subsequently counterstained with haematoxylin (HHS16, Sigma-Aldrich), dehydrated and mounted with DPX (06522, Sigma-Aldrich). Slides were examined through a microscope (IX71, Olympus) and images captured using the attached microscope camera (DP21, Olympus).

Statistical analysis

The statistical tests were clarified in related text or legends, all tests are two-sided unless otherwise specifically explained.

5.3 Results

5.3.1 Assessment of clinical parameters

Body Weight

As part of the clinical assessments, all mice of the four strains (WT, *Nlrp3*$^{-/-}$, Winnie, Winnie x *Nlrp3*$^{-/-}$) aged 12 weeks and 16 weeks of either sex were utilised for this study. The body weight of mice (n=10 per group) from all four genotypes were noted prior to sacrifice and the averaged body weight was calculated (WT 21.31 g, *Nlrp3*$^{-/-}$ 23g, Winnie 18.7g, Winnie x *Nlrp3*$^{-/-}$ 12wk 17.9g and Winnie x *Nlrp3*$^{-/-}$ 16wk 18.4g). All strains were then compared to WT strain (Figure 5.2). It was noted that the Winnie x *Nlrp3*$^{-/-}$ mice at 12 weeks exhibit significantly less (P-value <0.001) body weight relative to WT control.

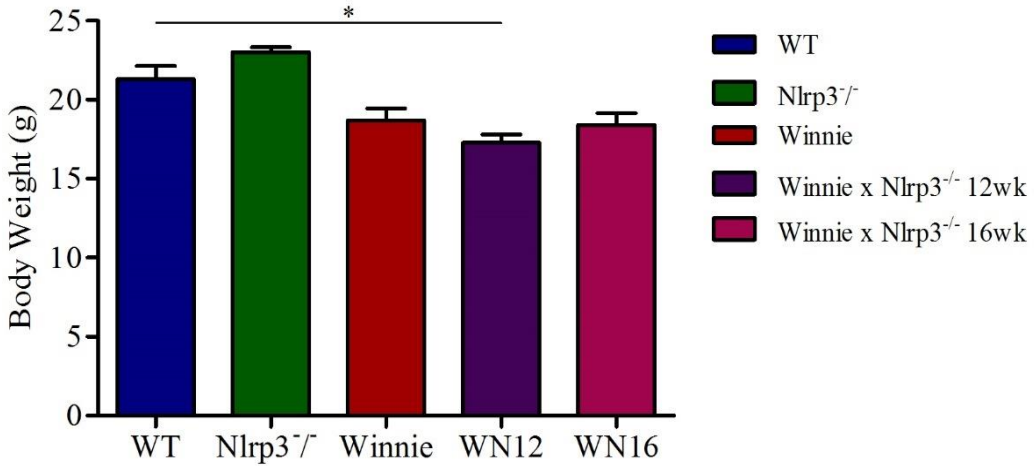


Figure 5.2: Mean Body weight (g) of the four experimental mouse strains. The data are representative of n=10 per group and are shown as the means±SEM. *P<0.05, (One-way ANOVA Dunn's Multiple comparison test).

Colon length

Colons from WT, Winnie, *Nlrp3*$^{-/-}$, and Winnie x *Nlrp3*$^{-/-}$ (n=12 per group), were retrieved and length was measured from the ileo-caecal junction up to the proximal rectum. Macroscopic observation of Winnie and Winnie x *Nlrp3*$^{-/-}$ colons showed the colons to be visibly inflamed with shortening and thickening of colon wall with enlarged mesenteric lymph nodes when compared to WT and *Nlrp3*$^{-/-}$ controls (Figure 5.3A).

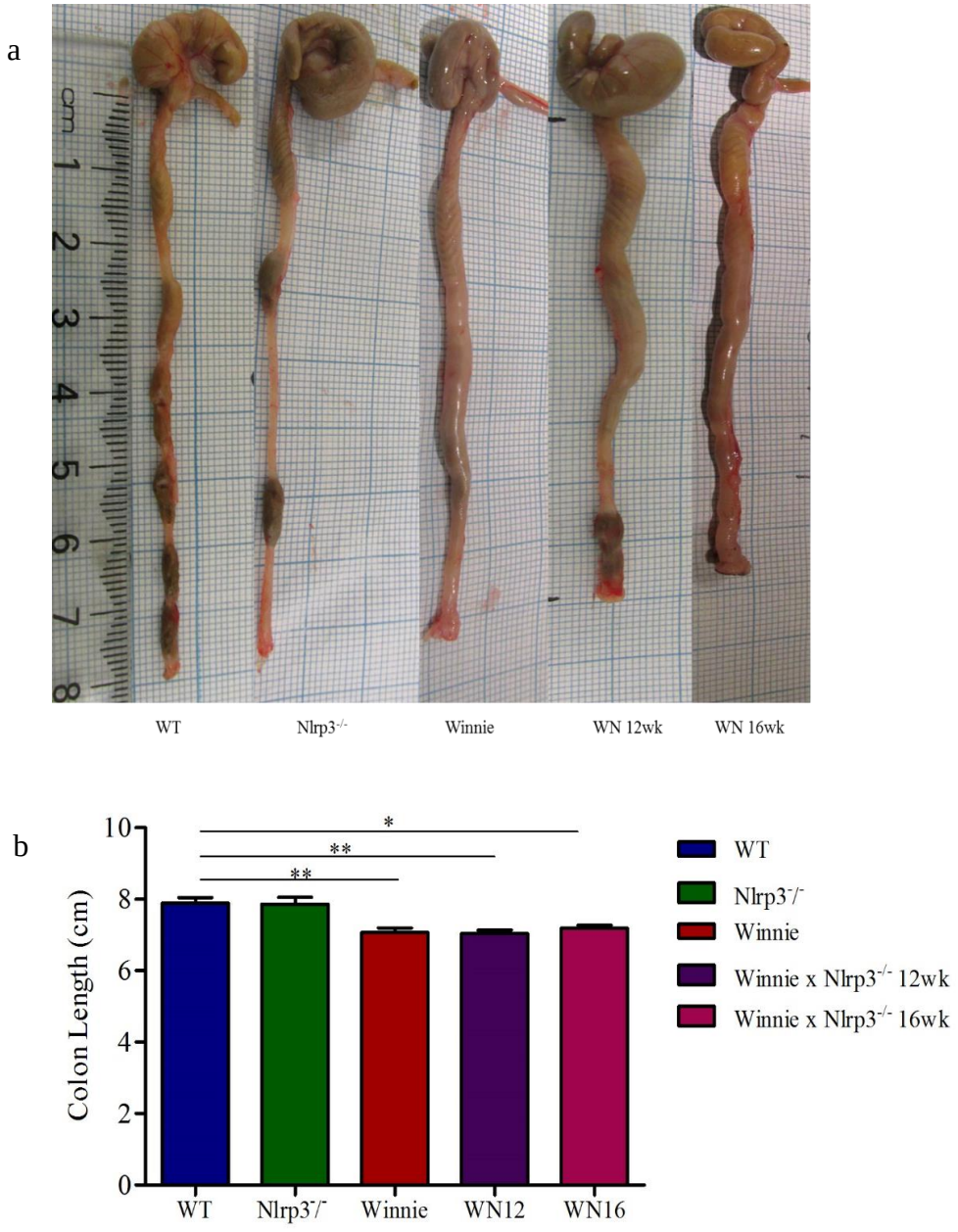


Figure 5.3: A. Colon images of the four experimental mouse strains from the cecum–colon junction to the anus end of a loosely stretched colon with a straight ruler. B. The data are representative of n=12 per group and are shown as the means±SEM. *P<0.05, **P<0.01 (One-way ANOVA Dunn's Multiple comparison test)

The mean colon length between the four genotypes were the following: WT–7.89 cm, Winnie –7.07 cm, *Nlrp3*$^{-/-}$ – 7.86 cm, Winnie x *Nlrp3*$^{-/-}$ 12 week –7.04 cm and Winnie x *Nlrp3*$^{-/-}$ 16 week 18.4g-7.19 cm. The colon length was compared to WT control and results showed statistically significant shortening between Winnie at P-value <0.01, Winnie x *Nlrp3*$^{-/-}$ 12 week at P-value <0.01 and Winnie x *Nlrp3*$^{-/-}$ 16 week at a P-value <0.05. The mean colon length *Nlrp3* $^{-/-}$ showed increase colon lengthening, however it failed to achieve a statistical significance (Figure 5.3B).

Colon weight/Body weight

The wet colon was cut opened longitudinally; faecal contents were removed and weighed for all four experimental mice strains. Wet colon weight, an indicator of intestinal oedema and inflammation, was presented as the ratio of colon weight over body weight (g/g). The ratio was significantly increased for Winnie at P-value <0.01, Winnie x *Nlrp3*$^{-/-}$ 12 week and Winnie x *Nlrp3*$^{-/-}$ 16 week at a P-value <0.001when compare to WT control. *Nlrp3*$^{-/-}$ showed no significant change in the mean ratio of colon weight over body weight relative to WT control (Figure 5.4).

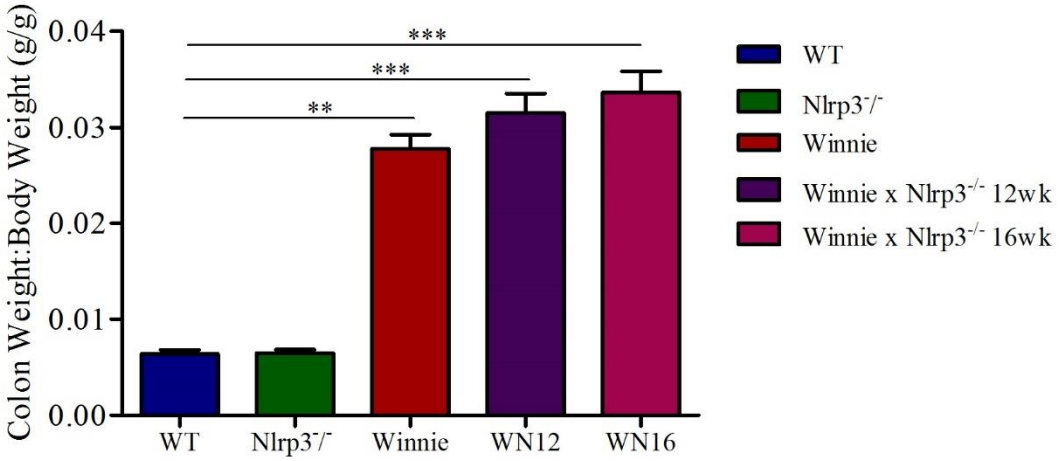


Figure 5.4: The mean colon weight/body weight ratio of the four experimental mouse strains. The data are representative of n=10 per group and are shown as the means±SEM. **P<0.01, ***P<0.001 (One-way ANOVA Dunn's Multiple comparison test)

Tumours per colon

Macroscopic assessment of open colon of WT and *Nlrp3*$^{-/-}$ showed healthy appearance with no sign of polyps. Winnie open colon appeared to have mucosal oedema and thickening of colon wall as seen described in previous studies (Heazlewood et al., 2008). We next examined the impact of the deficiency of NLRP3 activation on colon polyps, a

measurement of precancerous growth. We assessed the macroscopic polyp counts of the Winnie x *Nlrp3* $^{-/-}$ mice at week 12 and week 16 (Figure 5.5 A). Winnie x *Nlrp3*$^{-/-}$ mice at week 12 had severe mucosal oedema and precancerous polyps throughout the length of the colon and enlargement of mesenteric lymph nodes while at week 16 mucosal oedema had subsided with well-formed solid tumours throughout the length of the colon. Winnie x *Nlrp3*$^{-/-}$ mice at week 16 had a significantly higher (P-value <0.01) tumour load when compare to Winnie x *Nlrp3*$^{-/-}$ mice at week 12 (Figure 5.5 B).

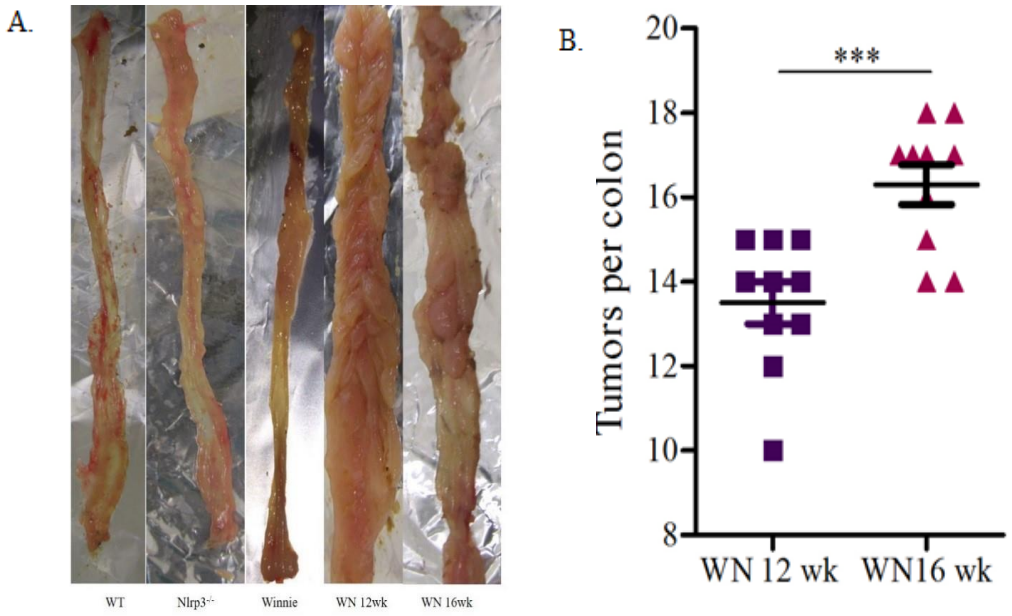


Figure 5.5: A. Representative images of the open colon of the four experimental strains at respective ages. B. The mean of macroscopic tumour counts for Winnie x *Nlrp3* $^{-/-}$ 12wk and Winnie x *Nlrp3* $^{-/-}$ 16wk mice strains. The data are representative of n=10 per group and are shown as the means±SEM. ***P<0.001 (Two-tailed Student's t test)

Survival analysis

The survival rates for the four experimental phenotypes were observed for period of 150 days. During this period WT and *Nlrp3*$^{-/-}$ had 100% survival. Winnie mice with chronic severe colitis are prone to develop rectal prolapse and it is reported to occur at any time between 9–20 weeks (Rahman et al., 2016). Winnie prolapsed mice are monitored closely and when body weight loss is > 15% were immediately and humanely euthanized. In the assessment we observed 70% survival rate of the Winnie strain. The novel model Winnie x *Nlrp3*$^{-/-}$ had a dramatic increase in the number of moribund with a survival rate of only 30% at the end of 150 days (Figure 5.6). These mice developed rectal prolapses at higher and early time point (6 weeks) than the Winnie strains and was humanely euthanized.

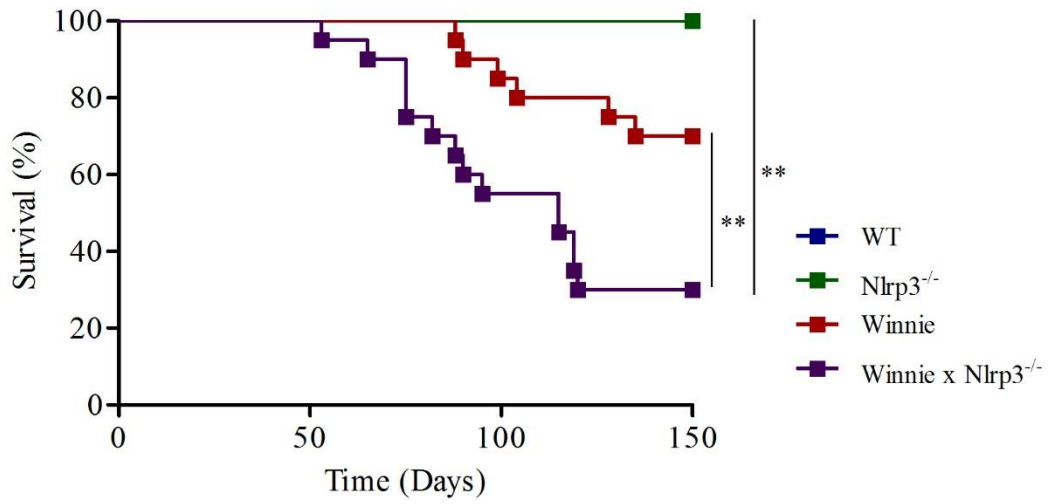


Figure 5.6: Kaplan-Meier survival analysis of Winnie and Winnie x *Nlrp3*$^{-/-}$ mice during an observation period of 150 days (n=20, per group). Survival rate, **P < 0.01 (Logrank (Mantel–Cox) test)

5.3.2 Histopathological assessment

Histopathological assessments were performed by me and a senior pathologist in a blinded fashion for H&E stained colon tissue sections of all four experimental strains using the grading system outlined in Table 5.2. The colon tissue section slides were examined and scored for histological hallmarks of inflammation, hyperplasia, low grade dysplasia, high grade dysplasia and invasive carcinoma. The WT mice colon and the *Nlrp3*$^{-/-}$ mice colon, when examined, did not indicate any inflammation or tumorigenesis and appeared to be normal which reflected in a histological score of zero. Thus, these two strains were subsequently excluded from the figure and statistical analysis.

Separate histopathological scorings for proximal (PC), mid (MC) and the distal (DC) colon regions were performed to determine whether spatial differences in pathology could be observed. The examination of H&E stained tissue sections revealed Winnie x *Nlrp3*$^{-/-}$ colon at 12 weeks had increased inflammation, hyperplasia, high grade dysplasia and invasive carcinoma at a non-significant increase in the proximal colon (PC) and mid colon (MC) and highly significant increase in the distal colon (DC) (P-value < 0.001) relative to Winnie PC, MC and DC (Figure 5.7). Winnie x *Nlrp3*$^{-/-}$ colon at 16 weeks had more dramatic statistically significant (P-value < 0.001) increase in inflammation, hyperplasia, high grade dysplasia and invasive carcinoma in the MC and DC (P-value < 0.001) relative to Winnie MC and DC and non-significant increase in PC relative to Winnie PC (Figure 5.7).

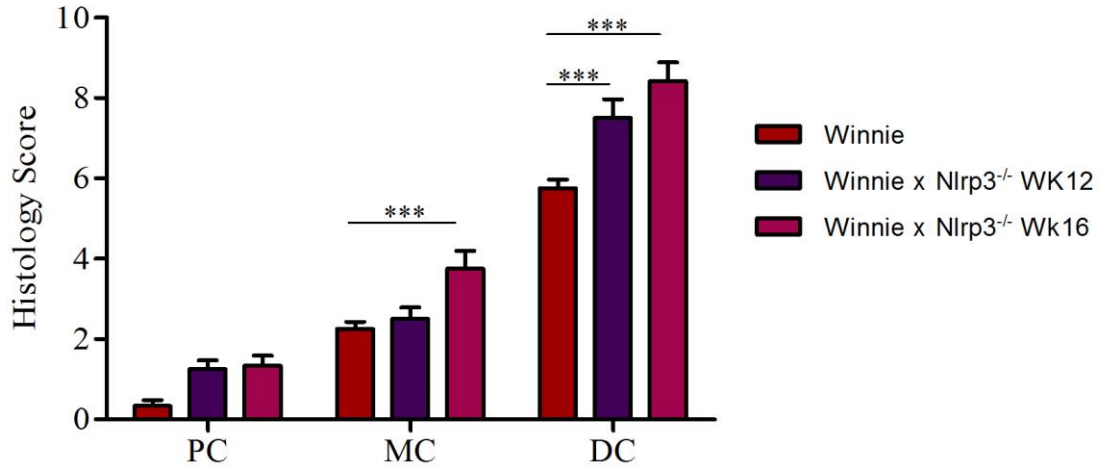


Figure 5.7: Histological score distribution in Wild-type, *Nlrp3*$^{-/-}$, Winnie and Winnie x *Nlrp3*$^{-/-}$ 12 wk. and 16wk colonic tissue. H&E stained colon tissue sections were graded using a scoring system for CAC. Combined scores from the proximal colon (PC), mid colon (MC) and distal colon (DC) segments are represented in the figure. The data are representative of n=12 per, group and are shown as the means±SEM. ***P<0.001 (Two-way ANOVA Bonferroni post-test)

This could be explained by the increase in polyp load at week 16 and associated significant increase in colon hyperplasia, dysplasia, in area involved with tumour pathology. Histopathological scoring for CAC markers agrees with significantly increased disease progression.

The PC of Winnie mice at 12 weeks exhibited increased infiltration of neutrophils, inflamed oedema, severe superficial epithelial damage and changes in crypt architecture, and mild hyperplasia indicative of active intestinal inflammation (Figure 5.8c). The inflammatory markers were significantly increased in distal colon of Winnie (Figure 5.8c). However, no incident of dysplasia or adenocarcinoma is reported in Winnie mice up to at 12 twelve months of age and similarly we did not observe these histological pathologies in the experimental Winnie mice.

100% of Winnie x *Nlrp3*$^{-/-}$ mice colonic mucosa displayed hyperplasia (Figure 5.1.0. b, c) and dysplasia (Figures 5.1.1, 5.1.2). Winnie x *Nlrp3*$^{-/-}$ mice showed hyper chromatism, complex glands sections, fibrinogen deposition (Figure 5.1.2.a) and flat dysplasia and individual gland dysplasia (Figure 5.1.3.b). Winnie x *Nlrp3*$^{-/-}$ colon displayed extensive ulceration sections and fibrinogen deposition which identify with precancerous lesions as seen in (Figure 5.1.0 a, b). We hypothesized that these structural abnormalities will lead to invasive carcinoma with progression of CAC. As predicted twenty percent of Winnie x

Nlrp3$^{-/-}$ at 12 week showed invasive carcinoma while this increased to forty percent at 16 weeks. (Table 5.1).

Table 5.1: Incidence of colitis associated dysplastic lesions and cancer.

Group	Non-Hyperplastic	Hyperplasia	Dysplasia	Invasive Carcinoma
C57BL/6J (12 week)	10/10 (100%)	0/10 (0%)	0/10 (0%)	0/10 (0%)
Nlrp3$^{-/-}$ (12 week)	10/10 (100%)	0/10 (0%)	0/10 (0%)	0/10 (0%)
Winnie (12 week)	2/10 (20%)	8/10 (80%)	0/10 (0%)	0/10 (0%)
Winnie x *Nlrp3*$^{-/-}$ (12 week)	0/10 (0%)	10/10 (100%)	10/10 (100%)	2/10 (20%)
Winnie x *Nlrp3*$^{-/-}$ (16 week)	0/10 (0%)	10/10 (100%)	10/10 (100%)	4/10 (40%)

Table 5.2: Scoring sheet of parameters for assessing H&E sections of colonic tissue.

Histology score sheet for NLRP3 x Winnie Colitis model

Slide no:

	Proximal Colon	Mid Colon	Distal Colon	Rectum
Inflammation				
0= Normal				
1 = mild (small, focal, or widely separated, limited to lamina propria)				
2 =moderate (multifocal or locally extensive, extending to submucosa)				
3 = severe (transmural inflammation with ulcers covering >20 crypts)				
Hyperplasia				
0= Normal				
1 = mild (some areas with crypts elongated and increased mitoses)				
2 =moderate (multifocal areas with crypts elongated up to twice the normal thickness, normal goblet cells present)				
3 = severe(mucosa twice the normal thickness, marked hyperchromasia, crowding or stacking, absence of goblet cells, high mitotic index and arborization)				
Low Grade Dysplasia				
0= Normal				
1 = occasional area				
2 = some areas				
3 = Many areas				
High Grade Dysplasia				
0= None				
1 = occasional area				
2 =some areas				
3 = Many areas				
Invasive Carcinoma				
0 = normal (0% involvement)				
1 = mild (up to 30% involvement)				
2 = moderate (30%–70% involvement)				
3 = severe (over 70% involvement).				

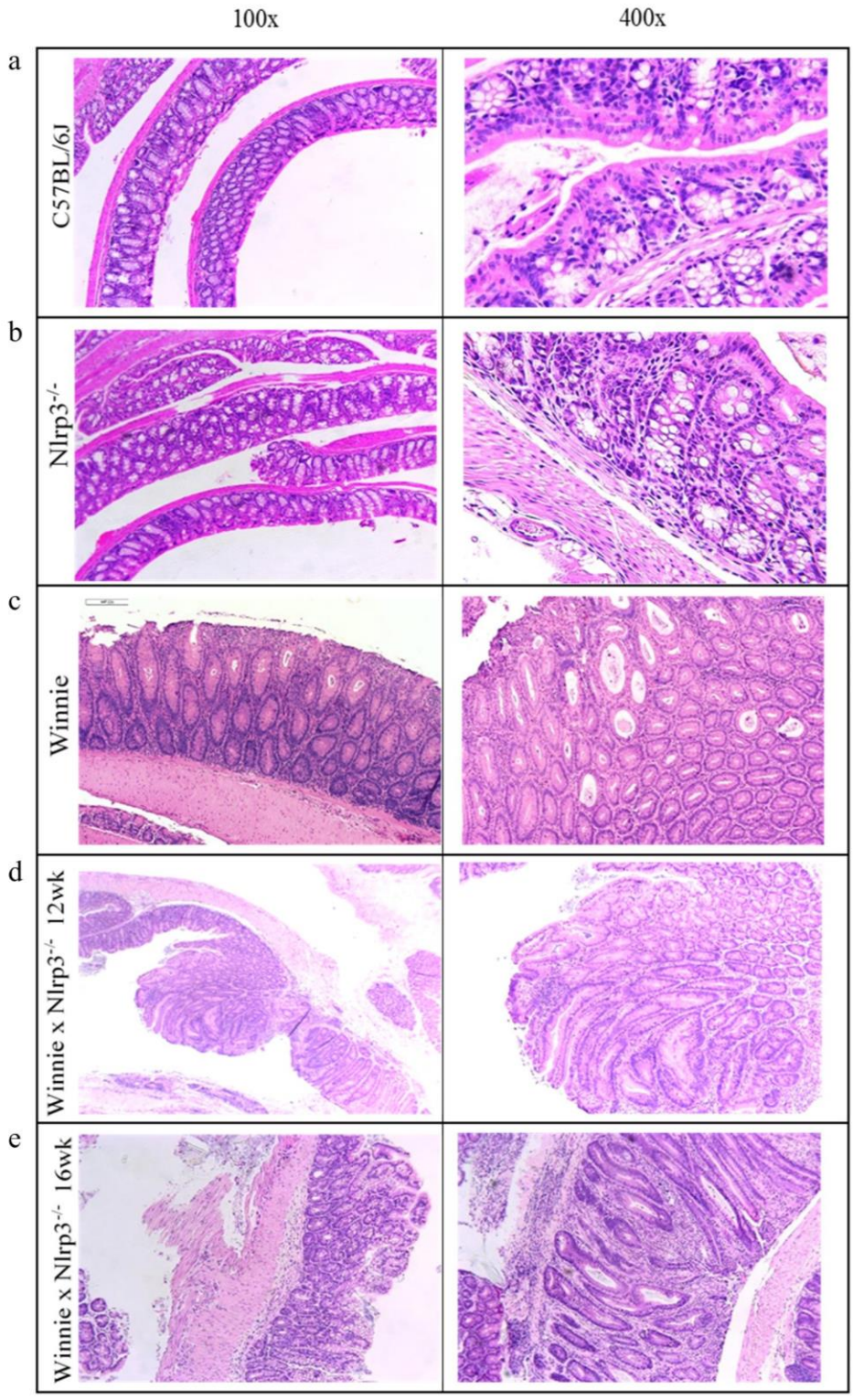


Figure 5.8: Comparative proximal colon histology from all genotypes. Representative H&E sections of proximal colon displaying colitis associated atypia in the proximal colon between a. Wild-type, b. *Nlrp3*$^{-/-}$, c. Winnie and d. Winnie x *Nlrp3*$^{-/-}$ 12 week. and e. 16-week mice. Magnification 100x and 400x.

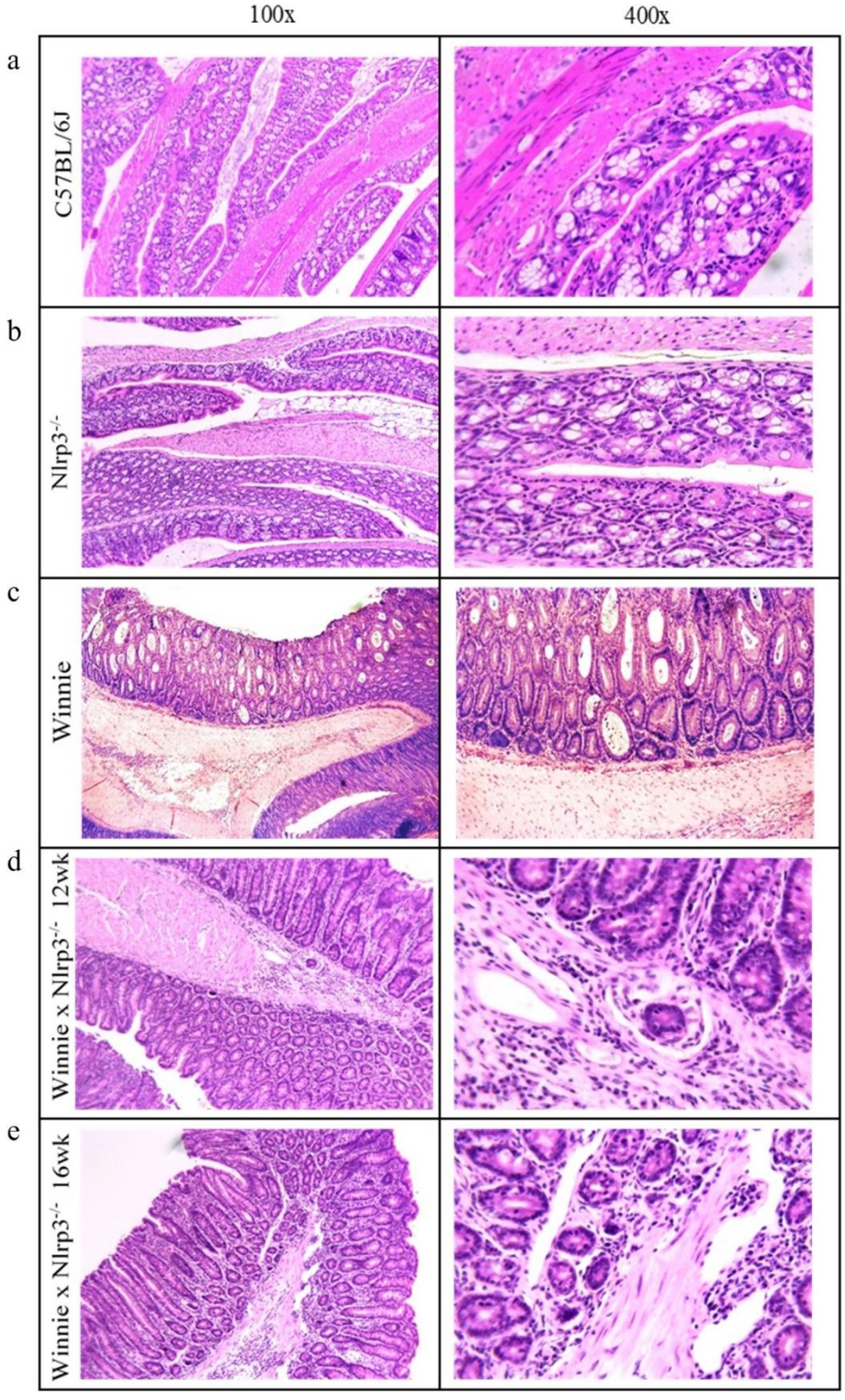


Figure 5.9: Comparative distal colon histology from all genotypes. Representative H&E sections of distal colon presenting the differences in colitis associated atypia in the distal colon between a. Wild-type, b. *Nlrp3*$^{-/-}$, c. Winnie and d. Winnie x *Nlrp3*$^{-/-}$ 12 week. and e. 16-week mice. Magnification 100x and 400x.

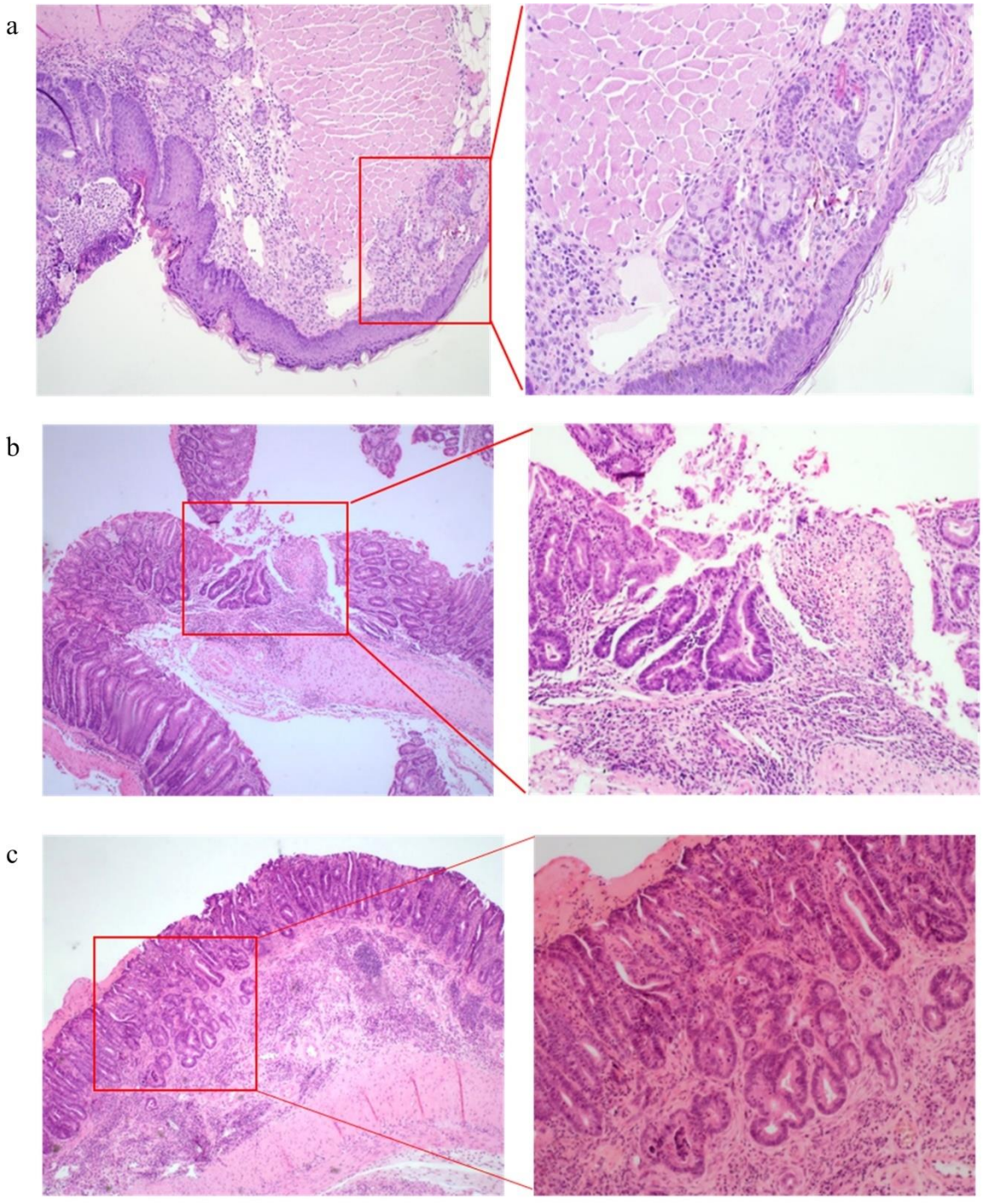


Figure 5.10: Histology of advanced colon cancer in Winnie x *Nlrp3*$^{-/-}$. a. High grade early invasive carcinoma b. Ulceration and hyperplasia and invasive carcinoma c. Invasive carcinoma observed in colons of Winnie x *Nlrp3*$^{-/-}$. Magnification is 100x and 400x.

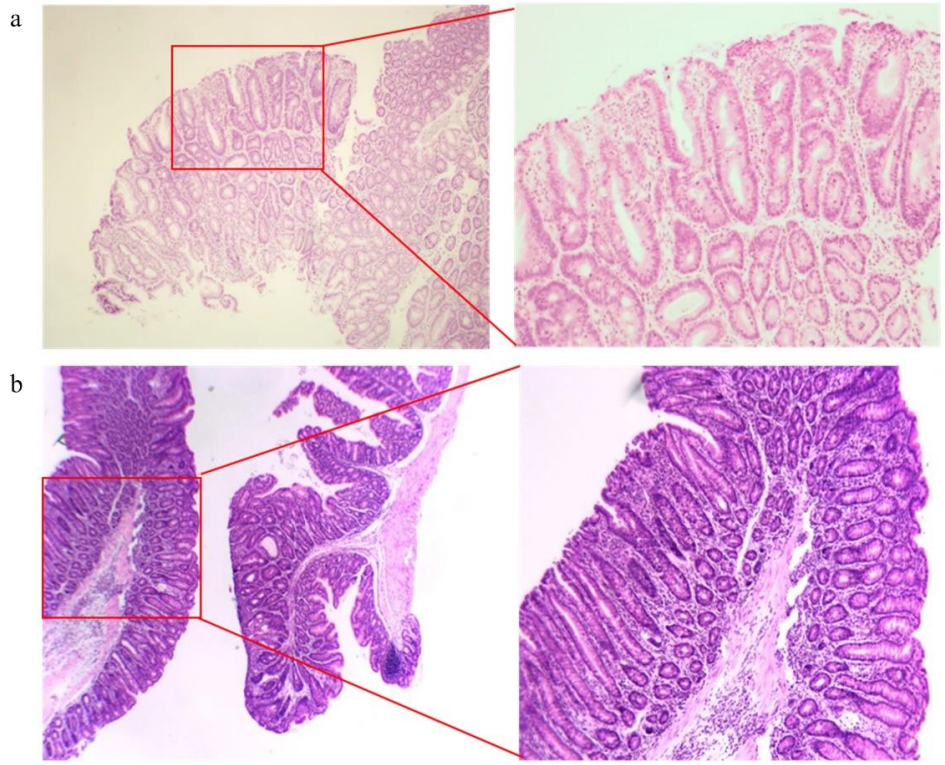


Figure 5.11: Representative images showing of H&E staining showing a. high grade dysplastic adenomatous polyp b. dysplasia and adenomatous polyploid in colons of Winnie x *Nlrp3*$^{-/-}$. Magnification 100x. and 400x.

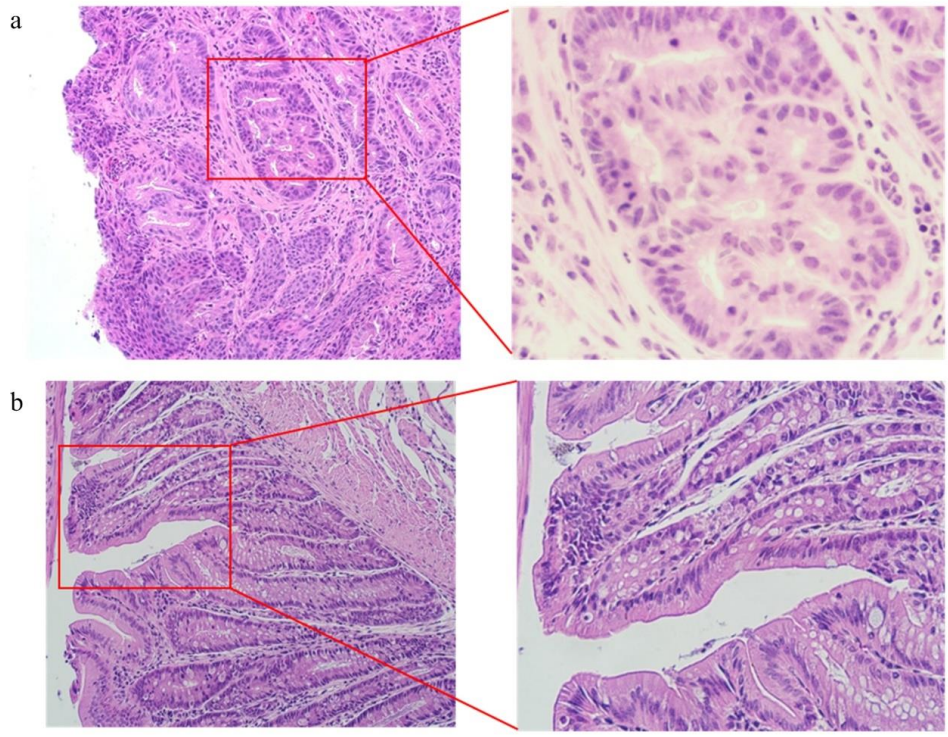


Figure 5.12: Representative images showing of H&E staining showing a. High grade dysplasia, hyper chromatism and complex glands b. dysplasia in colons of Winnie x *Nlrp3*$^{-/-}$. Magnification 100x. and 400x.

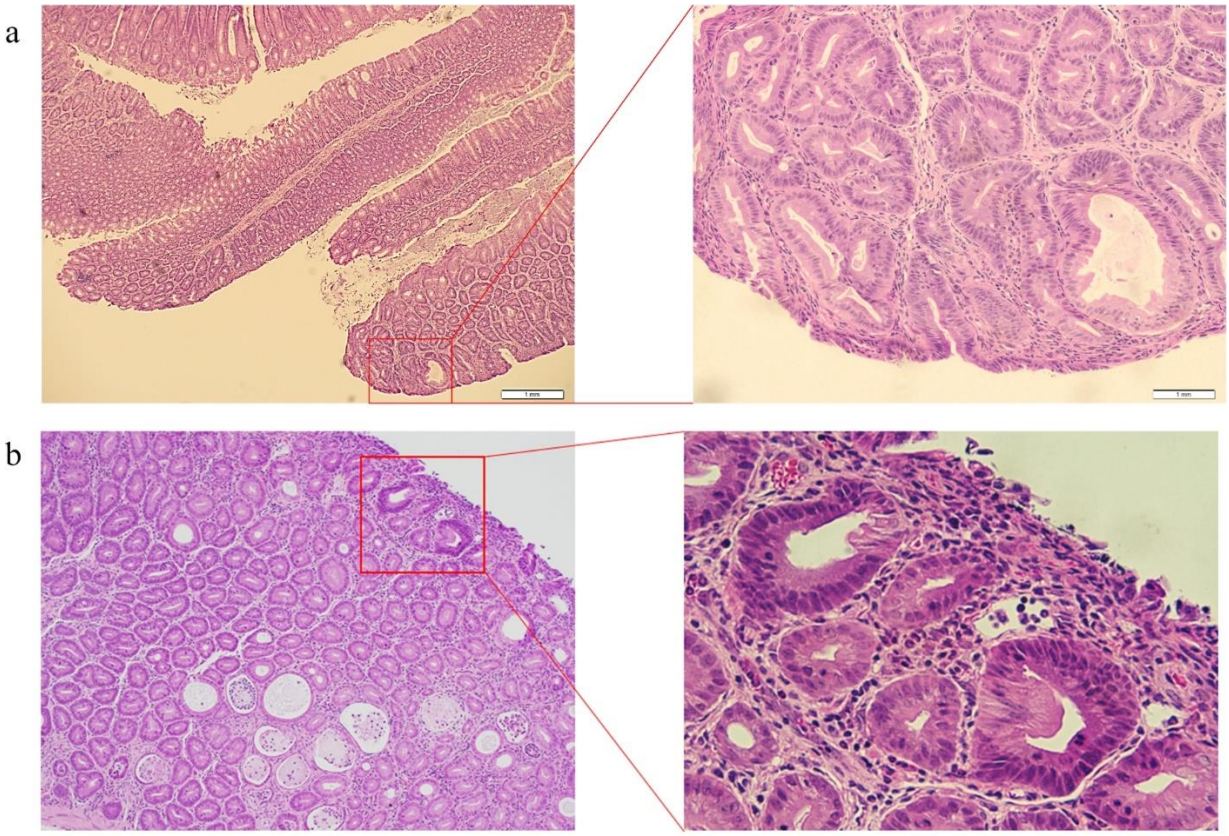


Figure 5.13: Representative images showing of H&E staining showing a. Flat dysplasia and complex glands b. Individual gland dysplasia, hyper chromatism and crypt abscess in colons of Winnie x *Nlrp3*$^{-/-}$. Magnification 100x. and 400x.

5.3.3 Biochemical Analysis

Inducible Nitric Oxide synthase activity

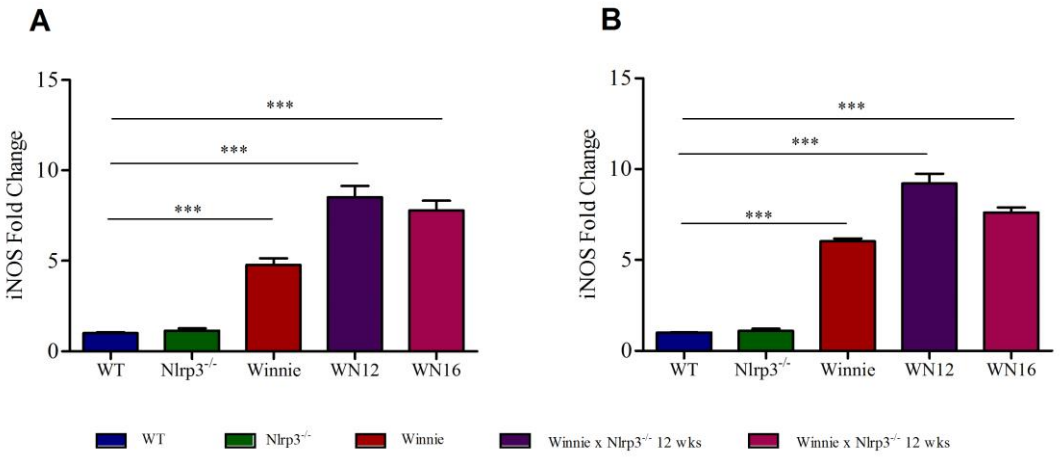


Figure 5.14: Nitric Oxide Synthase activity A. Proximal Colon B. Distal Colon presented as fold change as compared to the respective WT. The data are representative of three independent experiments and are shown as the means±SEM. ***P<0.001 (One-way ANOVA Dunn's Multiple comparison test)

The results show an increased fold change of iNOS concentration of the Winnie PC and DC relative to the WT control with a statistical significance of p<0.001. The novel CAC models Winnie x *Nlrp3*$^{-/-}$ at weeks 12 and 16 showed considerable increase of fold change in iNOS relative to WT at a statistical significance of p<0.001 (Figure 5.1.4).

Myeloperoxidase activity

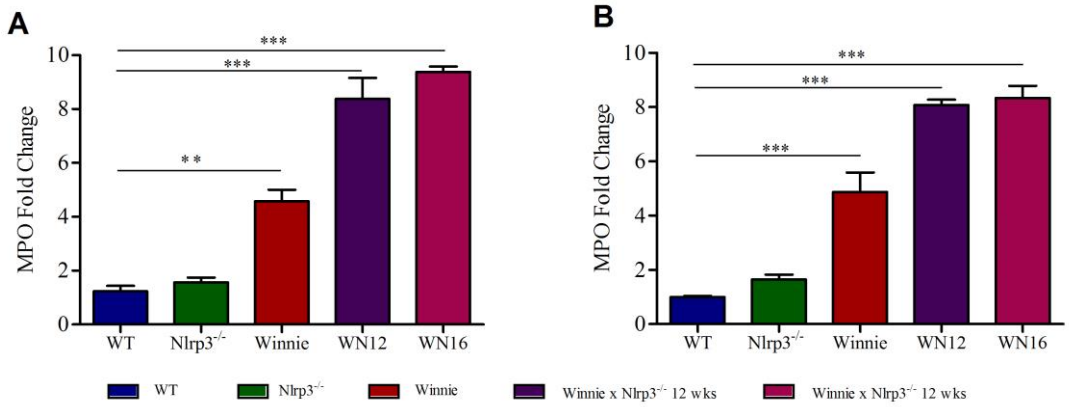


Figure 5.15: Myeloperoxidase activity A. Proximal Colon B. Distal Colon presented as fold change as compare to the respective WT. The data are representative of three independent experiments and are shown as the means±SEM. ***P<0.001 (One-way ANOVA Dunn's Multiple comparison test)

Colonic tissue of the four experimental groups were analysed for the concentration of MPO and compared to the WT control (Figure 5.1.5). The fold change of MPO concentration of the Winnie PC and DC relative to the WT control was respectively with a statistical significance of p<0.001. The novel CAC models Winnie x *Nlrp3*$^{-/-}$ at weeks 12 and 16 showed considerable increase of fold change in MPO relative to WT at a statistical significance of p<0.001.

Immunohistochemical analysis of DNA damage and oxidative stress markers

To investigate the oxidative stress associated DNA damage in the experimental models I looked at the protein expression of 8-Oxoguanine by colonic tissue immunohistochemistry (IHC). The highest expression was observed in the CAC model Winnie x *Nlrp3*$^{-/-}$ with medium expression in the colitis model Winnie and very little expression in WT control colonic tissue (Figure 5.17 a-c).

IHC was performed with the primary antibody anti- γH2A.X to look at the protein expression of γH2A.X in WT, Winnie and Winnie x *Nlrp3*$^{-/-}$ mouse colonic tissue. The highest expression was observed in the CAC model Winnie x *Nlrp3*$^{-/-}$ with medium

expression in the colitis model Winnie and very little expression in WT control colonic tissue (Figure 5.17 d-f).

To analyse the oxidative stress in the colon tissue, IHC was performed for NQO1 expression in WT, Winnie and Winnie x *Nlrp3*$^{-/-}$ mouse colonic tissue. The highest expression was observed in the CAC model Winnie x *Nlrp3*$^{-/-}$ with medium expression in the colitis model Winnie and very little expression in WT control colonic tissue. (Figure 5.17 g-I).

Additionally, I investigated another oxidative marker prominent in CAC, 3- nitrotyrosine in WT, Winnie and Winnie x *Nlrp3*$^{-/-}$ mouse colonic tissue. I observed highest expression in the CAC model Winnie x *Nlrp3*$^{-/-}$ with medium expression in the colitis model Winnie and very little to no expression in WT control colonic tissue (Figure 5.17 j-l).

C Reactive Protein

The serum CRP levels were analysed for all the colons of the experimental mouse models and the results showed a base level of 9.328 µg/ml in the WT control, elevated concentration of 16.81 µg/ml in Winnie and in Winnie x *Nlrp3*$^{-/-}$ 12 weeks at 18.88 µg/ml increasing to 24.66 µg/ml by 16 weeks.

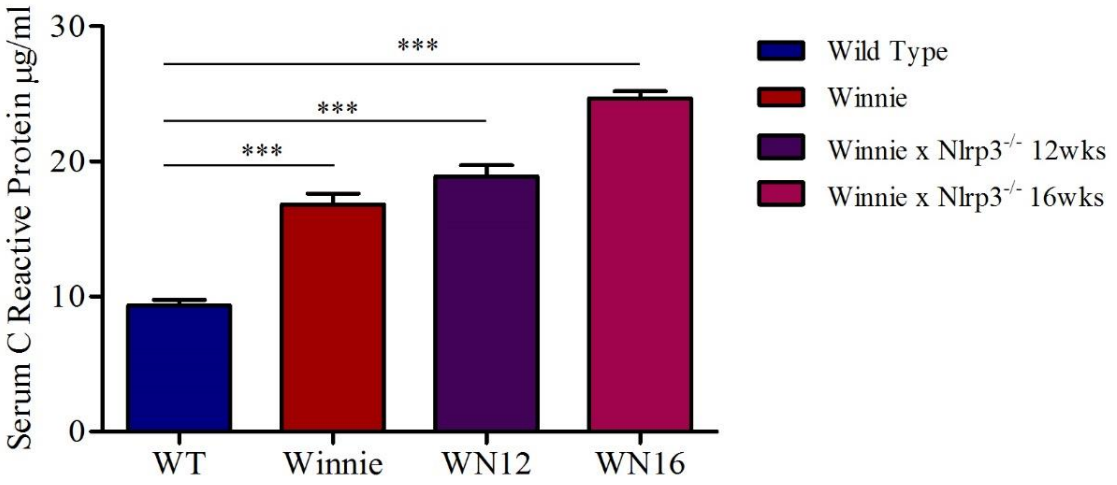


Figure 5.16: C Reactive Protein activity in serum. The data are representative of three independent experiments and are shown as the means±SEM. ***P<0.001 (One-way ANOVA Dunn's Multiple comparison test)

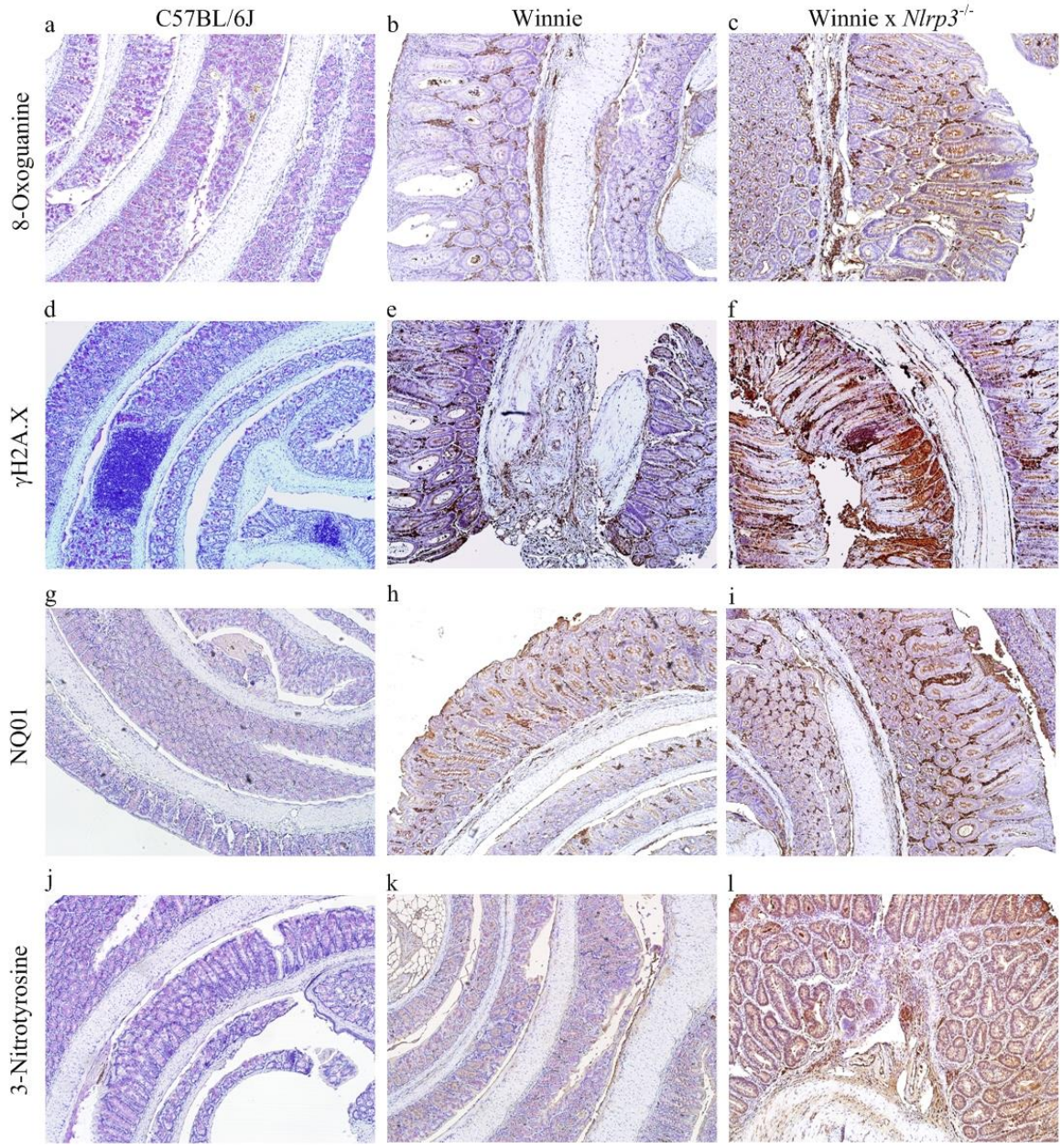


Figure 5.17: Immunohistochemical staining results for proteins 8-Oxoguanine (a, b, c), γH2A.X (d, e, f) NQO1 (g, h, i) and 3-Nitrotyrosine (j, k. l) in 12-week-old Wild Type, Winnie and Winnie x Nlrp3-/- mouse colonic tissue

5.3.4 Cytokine Profile

We investigated the cytokine profile of *Nlrp3*$^{-/-}$, Winnie and Winnie x *Nlrp3*$^{-/-}$ at 6wks, 12 weeks and 16 weeks and compared the levels to expression in WT. We did not observe differential production of cytokines between *Nlrp3*$^{-/-}$ and WT.

Instead, production of the NLRP3 inflammasome-associated cytokine IL-1β , IL-18 and IL-1α and inflammasome-independent cytokines IL-6, IL-17, GM-CSF, IL-12,IL-10 and

IFN-γ (Figure 5.1.8) and the chemokines KC, MCP-1 (also known as CCL2) and MIP-1α (also known as CCL3) was elevated in colon tissue of Winnie and Winnie x *Nlrp3*$^{-/-}$ compared to WT mice (Figure 5.1.9). We further confirmed these results and found statistically significant increased levels of circulating IL-1β, IL-6, TNF-α, IL-17 and G-CSF in the sera of Winnie x *Nlrp3*$^{-/-}$ compared to Winnie (Figure 5.2.0).

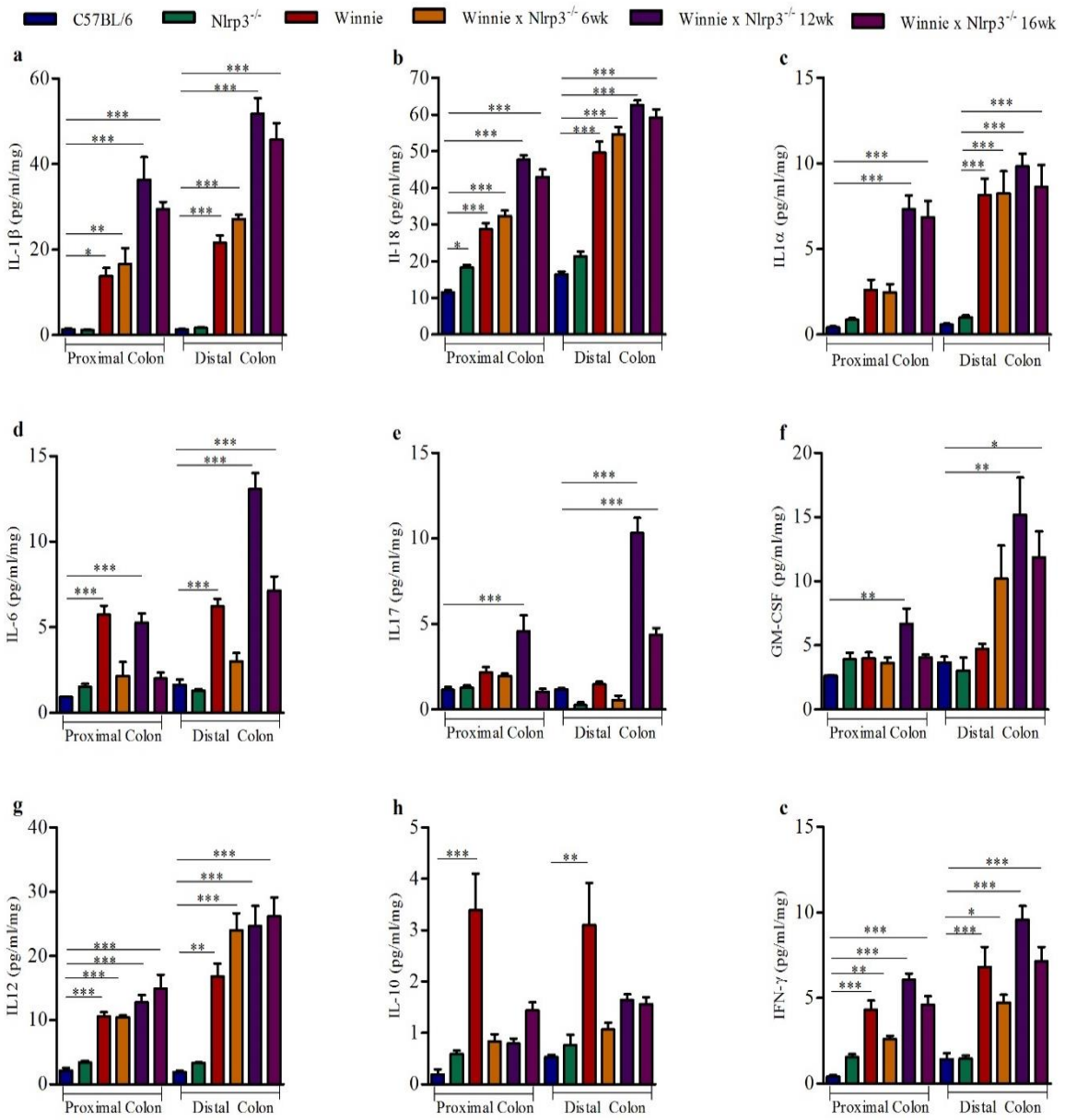


Figure 5.18: The indicated cytokines in the culture supernatants of colon tissues from mice. The data are representative of three independent experiments and are shown as the means±SEM. *P<0.05, **P<0.01, ***P<0.001 (One-way ANOVA Dunnett's Multiple comparison test)

The cytokines and chemokines showed an increase associated with age in Winnie x *Nlrp3*$^{-/-}$ at 6wks and 12 weeks however, a decrease in proinflammatory cytokine levels was observed in the Winnie x *Nlrp3*$^{-/-}$ at week 16.

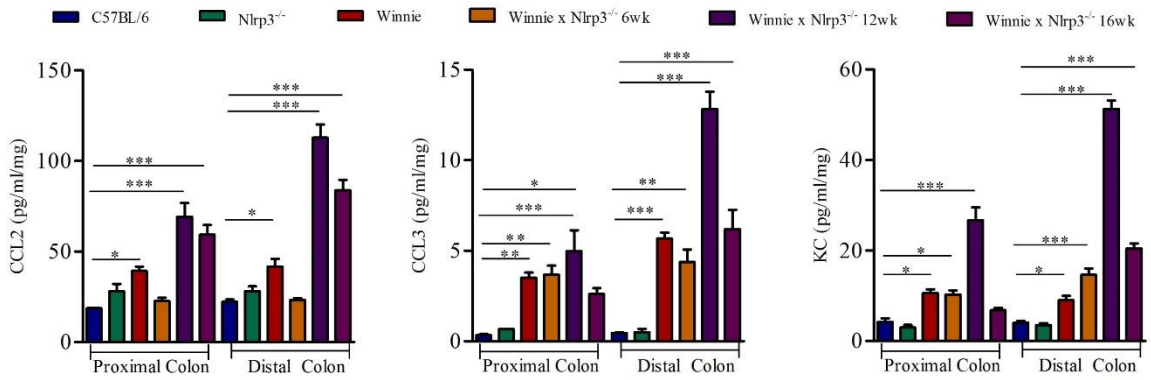


Figure 5.19: The indicated chemokines in the culture supernatants of colon tissues from mice. The data are representative of three independent experiments and are shown as the means±SEM. *P<0.05, **P<0.01, ***P<0.001 (One-way ANOVA Dunnett's Multiple comparison test)

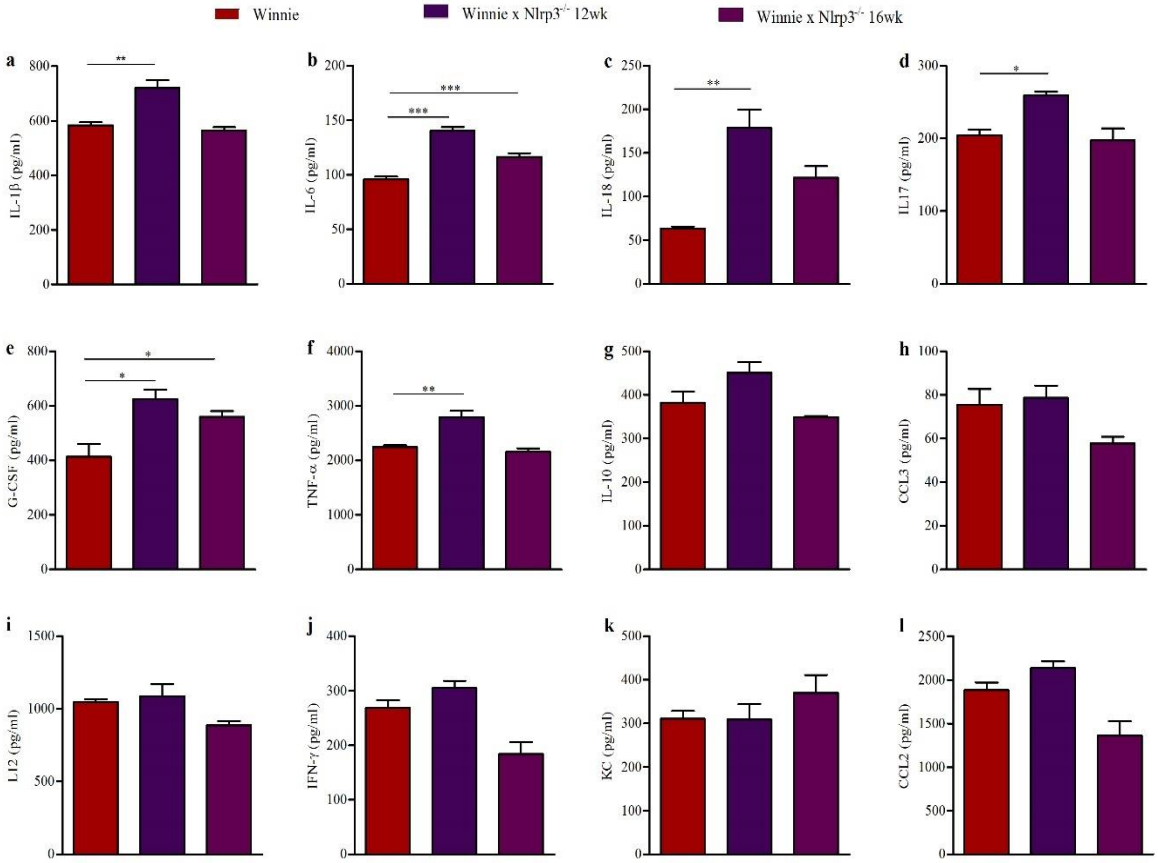


Figure 5.20: The indicated cytokines and chemokines in the blood serum from mice. The data are representative of three independent experiments and are shown as the means±SEM. *P<0.05, **P<0.01, ***P<0.001 (One-way ANOVA Dunnett's Multiple comparison test)

5.4 Discussion

Macroscopic observations of the colon of Winnie x *Nlrp3*$^{-/-}$ revealed all mice had noticeably inflamed and thickened colonic wall, enlargement of mesenteric lymph nodes, shortened colon length and formation of intestinal tumours compared to the Winnie control. This was a very interesting finding as no tumours are reported in Winnie mice up to twelve months of age (Randall-Demllo et al., 2016). Collectively, the macroscopic results indicated that a functional NLRP3 inflammasome is critical for protection against colitis associated dysplasia and tumorigenesis in the gut.

Examinations of colon histopathology from the novel model Winnie x *Nlrp3*$^{-/-}$ revealed all mice had increased mucosal inflammation, hyperplasia, dysplasia and polyps compared to Winnie controls. Thus, the deletion the *Nlrp3* gene in a colitis model has exacerbated colitis to colitis associated colon cancer phenotype through the classical sequence of no dysplasia progressing to indefinite dysplasia, low-grade dysplasia, high-grade dysplasia and eventually to submucosal invasion by the carcinoma. The mucosal tumours were assessed on established criteria such as structural and cellular atypia, alterations in tubular arrangement, abnormal epithelial cell proliferation and accordingly classified to low-grade or high-grade dysplasia or well-differentiated adenocarcinoma. The polyps were not focal but throughout the colon length as observed in CAC. The histopathological analysis revealed that many of the polyps were adenocarcinomas with a few advancing to invasive carcinomas. Macroscopic analysis of Winnie x *Nlrp3*$^{-/-}$ mice showed no distant metastases in other organs however, this was not experimentally analysed.

The histopathology of Winnie x *Nlrp3*$^{-/-}$ indicates that a functional NLRP3 inflammasome is critical for protection against tumorigenesis in a colitis disease state. Other studies support our findings however they have investigated in chemically induced DSS/AOM models (Zaki et al., 2011c, Allen et al., 2010). The novel model Winnie x *Nlrp3*$^{-/-}$ shows biological features like colitis associated colorectal dysplasia–invasive carcinoma sequence observed in human CAC and in future will be a useful experimental model for anti CAC therapeutic agents.

A hall mark of UC colonic inflammation is the infiltration and enhanced activity of phagocytic leukocytes leading to a production of excessive reactive oxygen species (ROS) and reactive nitrogen species (RON). These free radicals lead to oxidative/nitrosative stress

and cellular DNA damage which induces epigenetic mutations in genes related to cell proliferation, cell survival and stress induced pathways in colonic epithelial cells promoting tumorigenesis (Roessner et al., 2008, Waldner and Neurath, 2015). Inhibition of ROS and RON has been shown to effectively decrease DNA damage and delay (Hussain et al., 2003, Shaked et al., 2012).

The enzyme inducible nitric oxide synthase (iNOS) is highly expressed in inflammatory reactions. It is regulated by the transcription factor NF-κB, a known promoter of CAC. It is responsible for the generation of nitric oxide and is associated with the pathogenesis of CRC (Sawa and Ohshima, 2006). An increased expression of iNOS is associated with the mutagenesis of the tumour suppressor protein p53 and was found at an increased level in the colons of UC patients (Hussain et al., 2000). Analysis of human biopsies of colitis and CAC showed elevated iNOS protein expression levels (Gochman et al., 2012, Keshavarzian et al., 2003). In line with these studies our results show an increased fold change of iNOS concentration in the colitis model Winnie and CAC model Winnie x *Nlrp3*$^{-/-}$ when compared to WT mice.

Another inflammatory marker associated with CAC and abundantly found in colorectal tumours in human patients is myeloperoxidase (MPO) (Roncucci et al., 2008). MPO is an enzyme contained in lysosomes of neutrophils, monocytes and macrophages, active immune cells which infiltrate and accumulate in inflamed colonic mucosa (Krawisz et al., 1984). MPO is released upon activation of these immune cells converting hydrogen peroxide to generate cytotoxic oxidants increasing ROS in inflamed tissue. The results show a significant increase in MPO in the colonic tissue of Winnie and Winnie x *Nlrp3*$^{-/-}$ in comparison to WT. The increase in fold change observed in the spontaneous colitis model Winnie is consistent with other studies where they show an elevated expression of MPO pointing to enhanced neutrophil activity in UC (Krawisz et al., 1984, Raab et al., 1992). In agreement with the results in CAC model Winnie x *Nlrp3*$^{-/-}$ and other studies have shown MPO elevation in colorectal carcinomas and linked with increase in DNA damage (Roncucci et al., 2008, Hirt et al., 2013).

Reactive Oxygen and Nitrogen species (RONS) such as hydrogen peroxide, nitric oxide and superoxides leads to oxidative stress in inflamed tissues. These RONS interact with the DNA of resident cells and induce various forms of DNA damage including such as double-strand breaks (Jackson and Loeb, 2001). Oxidative stress inactivates mismatch repair

system, leading to the insertion of mutation into key oncogenes or tumour-suppressor genes contributing to tumour initiation (Colotta et al., 2009). Moreover, chronic inflammation induces higher rates of mitosis accelerating epithelial cell turnover in the colonic mucosa predisposing the mucosa to mutagenic assault resulting in DNA damage.

One of the major oxidative DNA damage biomarkers is 8-Oxoguanine, which is a mutagenic oxidative damage product of guanine. Guanine is the main target for reactive oxygen species in DNA, with 8-oxoguanine being the most frequent base lesion. Formation of 8-oxoguanine is common in inflamed and dysplastic tissue, but not in healthy mucosa (D'Inca et al., 2004). Recognition and removal of 8-oxoguanine is mediated by OGG1 DNA glycosylase which is elevated in CRC patients however it is insufficient to counteract the production of DNA damage associated with oxidative stress (Obtułowicz et al., 2010). Agreeing with these studies a high expression of 8-oxoguanine was detected in the Winnie x *Nlrp3*$^{-/-}$ colonic tissue.

DNA damage activates the DNA damage response (DDR) mechanisms such as direct repair and nucleotide excision repair (Curtin, 2012). One of the essential DDR to DNA double strand breaks (DSB) is the phosphorylated H2A.X (γH2A.X) which promotes recruitment of DNA repair proteins to sites of DSBs (Rogakou et al., 1998, Paull et al., 2000). This leads to significant accumulation of γH2A.X molecules relative to the severity of the DNA damage. Thus, γH2A.X has been used as a sensitive marker for the presence of inflammation induced DSBs in cells and tissues (Rogakou et al., 1998). Elevated expression of γH2A.X have been reported in IBD (Risques et al., 2008, Sohn et al., 2012) and various human cancer cell lines including colon cancer (Sedelnikova and Bonner, 2006). γH2A.X was highly elevated in the Winnie x *Nlrp3*$^{-/-}$ colonic tissue.

Quinone oxidoreductase 1 (NQO1) function under normal conditions is to protect cells from oxidative stress (Li et al., 2015). Oxidative damage promotes transcription factor Nrf2 and activates transcription of NQO1. Recent studies have reported high expression of NQO1 in several human solid tumours such as lung, bladder and colorectal cancer (Mikami et al., 1998, Chao et al., 2006). Another study showed the high expression of NQO1 protein in CRC tissues compared with adjacent non-tumour tissues by immunohistochemistry staining (Ji et al., 2014). NQO1was highly expressed in the Winnie x *Nlrp3*$^{-/-}$ colonic tissue indicating a high level of oxidative stress in tumour associated tissue.

The conversion of tyrosine to 3-nitrotyrosine have been reported to be through the nitrating agent peroxynitrite a key component of the nitric oxide signalling pathway (Ischiropoulos et al., 1992). Analysis of human biopsies of colitis and colon cancer using immunohistochemistry revealed elevated iNOS and nitro tyrosine protein expression levels in these tissues (Gochman et al., 2012). Another study reported observation of nitro tyrosine in a number of human cancer tissue including colorectal carcinoma (Kondo et al., 2002). Winnie x *Nlrp3*$^{-/-}$ colonic tissue showed an elevated level of iNOS and high expression of 3-nitrotyrosine indicating nitrosative stress due to CAC pathogenesis.

CRP analysis in Winnie x *Nlrp3*$^{-/-}$ mice showed an increase as compared to WT indicating the severity of CAC. C-reactive protein (CRP) is an acute phase protein produced primarily in the liver and is mainly induced by the proinflammatory cytokines IL-6 (Castell et al., 1990, Pepys and Hirschfield, 2003). Circulating serum value of CRP is a major clinical indicator of ongoing tissue inflammation and has been used as a reliable biomarker evaluating the disease activity and extent of inflamed tissue in UC patients (Solem et al., 2005, Chouhan et al., 2006). The chronic inflammation associated with colon tumour tissue initiates a systemic inflammatory response that can be measured by the circulating inflammatory marker CRP levels and has been used as a prognostic indicator for all stages of colorectal cancer (Roxburgh et al., 2009, Contu et al., 2009). Studies have reported that the risk of mortality among patients with colorectal cancer is positively associated with plasma CRP (Cooney et al., 2013, McMillan et al., 2003).

Chronic intestinal inflammatory conditions such as IBD can lead to CAC (Rubin et al., 2012). Once tumours are generated, they are tightly controlled by the tumour microenvironment which is regulated by proinflammatory cytokines and chemokines released from epithelial and tumour-infiltrating immune cells. These proinflammatory mediators act on epithelial and premalignant cells activates molecular mechanisms of cell proliferation, angiogenesis and inhibition of apoptosis (Grivennikov and Karin, 2010b, Grivennikov and Karin, 2010a).

To elucidate the activated pathogenic molecular pathways in CAC, it is essential to identify and measure the proinflammatory cytokine levels in the diseased colon. Thus, multiple cytokines were assessed in colonic culture supernatants and the results were compared to WT control. The results showed that the *Nlrp3*$^{-/-}$ mice showed basal expression of cytokines

comparable to the WT control. This supports the idea that the NLRP3 inflammasome is redundant in a non-disease condition.

The key downstream effector molecules associated with NLRP3 inflammasome activation, IL-1β, IL-18 and IL-17 showed an increase in Winnie as previously reported (Eri et al., 2011). Winnie mice treated with the specific NLRP3 inhibitor MCC950 had a significantly low level of these inflammatory mediators as compared to untreated Winnie mice (Perera et al., 2018). However, interestingly the expression of these cytokines showed a significant age dependent increase in the Winnie x *Nlrp3*$^{-/-}$ model (6 week and 12 week) even in the absence of a functional NLRP3 inflammasome.

In CAC pathogenesis IL-1β acts as key protumorigenic factor by associating with massive infiltration of neutrophils and regulating proinflammatory cytokines and chemokines (Wang et al., 2014c). Over production of IL-1β in CAC, promotes differentiation of naive T cells in to Th17 cells that produces the proinflammatory cytokine IL-17 (Acosta-Rodriguez et al., 2007). IL-1β is not only linked to the induction of the tumour microenvironment but also initiates tumour invasiveness (Voronov et al., 2003, Krelin et al., 2007). It is now believed that NLRP3 and IL-1β are selectively expressed in the LPMCs while IL-18 is only expressed in intestinal epithelial cells (Zhu et al., 2017, Yao et al., 2017b). In line with this finding a recent study has shown the functional role of NLRP3 activation is carried out by elevated production of IL-1β and not IL-18 (Yao et al., 2017b). Thus, the high expression of IL-1β seen in Winnie x *Nlrp3*$^{-/-}$ colon could be the driving factor of CAC seen in this model.

Initial investigations on the role of NLRP3 in colitis and CAC models showed a reduced expression of IL-18 in NLRP3 deficient mice (Zaki et al., 2011b, Allen et al., 2010). During DSS-induced colitis in *Nlrp3*$^{-/-}$ mice, IL-18 was essential for the intestinal tissue repair of the ulcerated epithelium during the acute inflammatory phase and injecting recombinant IL-18 reduced the severity of colitis (Zaki et al., 2010b). In DSS/AOM induced CAC, IL-18 was found to be protective against colitis associated tumorigenesis by the activation of tumor suppressors STAT1 and IFN-γ (Zaki et al., 2010b). These results established IL-18, but not IL-1β, as the critical player for NLRP3 inflammasome-mediated resistance to colitis and CAC.

In contrast to the previously published studies the results of the spontaneous colorectal cancer model Winnie x *Nlrp3*$^{-/-}$ deficient in NLRP3 showed an increased expression of IL-18. This unexpected result could be explained by recent studies where it is shown that the epithelium caspase-1 activation and IL-18 secretion were found to be independent of NLRP3 (Yao et al., 2017b, Song-Zhao et al., 2013). Moreover, the Winnie x *Nlrp3*$^{-/-}$ model is not chemically induced CAC and compared to AOM/DSS model it has a more intact epithelium with better ability to produce IL-18 as compared to the chemically eroded epithelium in DSS model. Additionally, during colitis and associated colorectal cancer infiltrating macrophage cells will also contribute to IL-18 production (Zaki et al., 2010b). It is reported that higher concentration of IL-18 promotes tumorigenesis (Siegmund, 2010a). Finally, IL-18 was shown to induce Fas ligand production and the generation of multiple secondary proinflammatory cytokines, chemokines, cell adhesion molecules, and NO species (Maxwell et al., 2006, Horwood et al., 1998). Thus, in the Winnie x *Nlrp3*$^{-/-}$ colon IL-18 plays a pro-tumorigenic role.

In a hypoxic tumour microenvironment undergoing high tissue damage and inflammation caspase-1 activation by inflammasomes leads to GSDMD-induced pyroptosis allowing the release of high doses of the alarmin IL-1α (Voronov et al., 2013, Gross et al., 2012). The bioactive extracellular IL-1α is mainly generated by the epithelial cells and is associated with driving inflammation at mucosal barrier surfaces (Malik et al., 2016). In recent years, IL-1α has also emerged as an important driver of colon inflammation and cancer (Bersudsky et al., 2014). Studies have shown that IL-1α deficient mice are protected from DSS induced acute colitis (Bersudsky et al., 2014, Malik et al., 2016). A study has shown carcinoma-derived IL-1α and IL-1β activates mesenchymal stem cells to produce Prostaglandin E2 and cytokines, such as IL-6, IL-8 and RANTES, which in turn, induce activation of β-catenin in the malignant cells and their transition into Cancer Stem Cells leading to tumour initiation (Li et al., 2012). Similarly, IL-1α is highly expressed in the Winnie x *Nlrp3*$^{-/-}$ colon and exacerbates colitis towards CAC.

Numerous studies have reported increased levels of IL-6 trans signalling play a crucial pathogenic role in IBD patients and is positively correlated with the severity of inflammation (Atreya and Neurath, 2008, Atreya and Neurath, 2005). In the Winnie x *Nlrp3*$^{-/-}$ model IL-6 peaked at week 12 with the appearance of intestinal tumours. Other studies also found elevated IL-6 in CAC patient serum and in the tumour

microenvironment and correlated with tumour size and disease status in various clinical and experimental studies (Komoda et al., 1998, Chung and Chang, 2003, Grivennikov and Karin, 2010a). In addition, IL-6 has been associated with liver metastases and reduced survival (Heikkila et al., 2008, Olsen et al., 2015). This can be attributed to LPMC ability to generate IL-6, to enhance proliferation of tumour progenitor cells and the protection of normal and premalignant intestinal epithelial cells from apoptosis by the signal transducer and activator of transcription 3 (STAT3) signalling (Becker et al., 2005, Huber et al., 2012). Recent studies have demonstrated that the IL-6/STAT3 axis as a crucial tumour promoter in colitis-associated cancer (Grivennikov et al., 2009, Bollrath et al., 2009). Prolonged STAT3 activation enhances the sustained expression of IL-6 induced proteins that play important roles in inflammation and carcinogenesis (Wang et al., 2013). Due to the correlation of IL-6 expression with CRC prognosis and the increased expression of IL-6 in patients with IBD, IL-6 could be the tipping point of chronic inflammation pathogenesis to tumour development in the Winnie x *Nlrp3*$^{-/-}$ model. Another key proinflammatory role of IL-6 is the differentiation of Th1 to Th17 cells, which is the major source of IL-17 (Bettelli et al., 2006, Atreya et al., 2000).

An increased level of IL-17 is observed in Winnie x *Nlrp3*$^{-/-}$ model and has been associated in active IBD (Fujino et al., 2003) as well as CAC pathogenesis (Wang et al., 2014a). The proinflammatory role of IL-17 in the pathogenesis of CAC was also confirmed in animal models. Blocking IL-17A attenuated colitis and reduced tumour burden in *APC*$^{min/+}$ mice and AOM/DSS treated mice (Hyun et al., 2012, Chae et al., 2010). Additionally, It is suggested that IL-17 facilitates angiogenesis and promotes CRC development by inducing the production of VEGF (Liu et al., 2011).

GM-CSF promotes CAC in AOM/DSS-challenged mice as treatment with a neutralizing anti-GM-CSF antibody decreased tumour development and colitis score in this model (Wang et al., 2014b). GM-CSF is produced by IECs and even more by neoplastic colonic epithelial cells (Nebiker et al., 2014, Wang et al., 2014b). Commensal microbiota derived LPS triggers GM-CSF expression in IECs (Wang et al., 2014b) which would explain the increase of GM-CSF observed in Winnie PC and DC. Stromal fibroblasts and lymphocytes adjacent to the CRC tumour have also been found to be positive for GM-CSF (Trutmann et al., 1998). Cancerous epithelial cells, monocytes, and antigen-presenting cells all express the GM-CSF receptor in the CRC microenvironment (Urdinguio et al., 2013, Nebiker et

al., 2014, Wang et al., 2014b). GM-CSF induces autocrine or paracrine VEGF release by IECs (Wang et al., 2014b), thereby promoting angiogenesis; yet, it does not have a direct proliferative effect on these cells (Trutmann et al., 1998). GM-CSF binding to its receptor activates the JAK-STAT, the MAPK, and the PI3K pathways, which results in cell survival and proliferation (Hercus et al., 2009). The high level of GM-CSF observed in Winnie x *Nlrp3*$^{-/-}$ could be driving colitis to CAC through its association with VEGF and PI3K pathway.

CAC progression is determined not only on pro-tumour mediators discussed so far but also with the anti-tumour factors activated by the immune response of the host. The clinical outcome of CAC patients depends on the balance between the pro-tumour and anti-tumour mediators at the invasive site of colorectal cancer.

IL-12 is crucial for the induction and the expansion of Th1 responses as well as the activation of cytotoxic immune effectors, such as NK and CD8+ T cells (Engel and Neurath, 2010). IL-12 exerts its anti-tumour effect by activating the IFN-γ production in these cells and subsequent generation of IFN-γ initiates antiproliferative, antiangiogenic, and cytotoxic effects that limits tumour growth (Nardin and Abastado, 2008, Hayes et al., 1995). In the Winnie x *Nlrp3*$^{-/-}$ we see an age dependent increase of IL-12 as observed in other CAC models.

IL-10 is an anti-inflammatory cytokine which suppresses IBD and CAC mouse models. IL-10 deficient mice are highly susceptible to UC and CAC due to uncontrolled inflammation in the colon and develop spontaneous adenocarcinoma of the colon by 6 months of age (Berg et al., 1996, Dennis et al., 2015, Sturlan et al., 2001). IL-10 deficient mice showed a marked increase CD4+ cells and macrophages in the lamina propria, along with associated elevated levels of TNFα, IL-6 and nitric oxide (Davidson et al., 1996). The anti-tumorigenic effect of IL-10 could be attributed to it limiting a pathogenic Th17 inflammatory response (Chaudhry et al., 2011). In agreement with these studies the IL-10 expression in Winnie x *Nlrp3*$^{-/-}$ age dependently decreases with progression of CAC.

IFN-γ is known to be produced by Th17 cells to induce pathogenic responses (McGovern and Powrie, 2007) however IL-18 has been shown to play the major role in the induction of IFN-γ production (Wong et al., 2013, Chaix et al., 2008, Takeda et al., 1998). IFN-γ subsequently activates several intrinsic cellular pathways such as Janus kinase (JAK)-STAT signalling (Zaki et al., 2011a). Activation of JAK-STAT signalling leads to nuclear

translocation of the STAT1 protein and increases the transcription regulation of IFN-γ induced genes, which are responsible for proliferation, differentiation, and cell death. In addition, IL-18-INF-γ-STAT1 axis activation is involved in colon carcinogenesis (Zaki et al., 2011b). Mechanistically, IFN-γ acts on CRC cells by inducing STAT1 phosphorylation and inhibiting the EGFR/Erk1/2 and Wnt/βcatenin signalling pathways, thereby restraining cell proliferation (Wang et al., 2015a). In line with these studies I observed an age dependent increase of IFN-γ in Winnie x *Nlrp3*$^{-/-}$.

Winnie x *Nlrp3*$^{-/-}$ mice showed poor survival with 50% survival rate at 16 weeks. Winnie x *Nlrp3*$^{-/-}$ mice colonic tissue and serum analysis showed an elevation of proinflammatory cytokines, MPO, iNOS, CRP and ROS levels. These molecules activated proinflammatory pathways aggravating colitis to CAC leading to a decrease in survival rate.

A decrease in most of the proinflammatory cytokine levels were observed in the Winnie x *Nlrp3* $^{-/-}$ at week 16. Macroscopic analysis at this age showed solid colonic tumours. These established tumours create a local environment with low levels of inflammation which can be immunosuppressive and is therefore favourable for tumour growth, angiogenesis, and metastasis.

5.5 Conclusion

The clinical assessment of the novel mouse model Winnie x *Nlrp3*$^{-/-}$ colon displayed aggravated inflammation and spontaneous tumorigenesis. Histopathological assessment of Winnie and Winnie x *Nlrp3*$^{-/-}$ mice revealed all the features of the classical pathway of CAC starting with high inflammation to hyperplasia, dysplasia and invasive carcinoma. The biochemical analysis quantified the excessive production of ROS resulting in elevated oxidative stress in Winnie x *Nlrp3*$^{-/-}$ mice colonic tumours. The immunohistochemical analysis showed high levels of oxidative stress markers present in the colorectal tumours and the inactivation of the DNA repair system by oxidative stress leading to genomic mutations in oncogenes and tumour suppressor genes initiating and promoting tumorigenesis in the colon.

The cytokine profile of Winnie x *Nlrp3*$^{-/-}$ mice was analysed to understand the underlying immunopathology. The colons of Winnie x *Nlrp3*$^{-/-}$ are highly inflamed by the generation of significantly elevated levels of cytokines and chemokines associated with CAC in the local tumour microenvironment as well as in serum. Moreover, even with deficiency of

NLRP3 inflammasome over production of IL-1β, IL-18 and IL-1α and subsequent activation of the adaptive immune system promotes an enhanced pro-inflammatory environment locally in Winnie x *Nlrp3*$^{-/-}$ initiating tumorigenesis in colonic tissue.

Taken together, Winnie x *Nlrp3*$^{-/-}$ represents human CAC, where the progress from chronic inflammation in UC to the morphological features of CAC initiates with constant production of pro-inflammatory cytokines, chemokines and reactive oxygen and nitrogen species promoting aberrant intestinal epithelial cell proliferation, survival and angiogenesis leading to epithelial dysplasia of low grade to high grade and progression to invasive carcinoma over time.

In summary, these data demonstrate ablating the *Nlrp3* gene in the spontaneous colitis Winnie mutant mouse leads to severe colorectal cancer. To our knowledge, this is the first study to identify such a mechanism for NLRP3 inflammasome in a spontaneous colitis mouse model similar to human UC. The findings thus emphasize the critical contribution of the NLRP3 inflammasome as a negative regulator of CAC.

Chapter 6

Molecular, Microbiota and metabolomic analysis of Winnie x *Nlrp3*$^{-/-}$ mouse colon

6.1 Introduction

Cancer cells are characterized by abnormal signalling pathways involving cell proliferation, apoptosis, and angiogenesis. These pathways have also been linked to chronic inflammation, a major mediator of tumour progression in several cancers. I analysed these pathways in the Winnie x *Nlrp3*$^{-/-}$ model to reveal the mechanism of colitis in to CAC.

In colitis associated carcinogenesis it is speculated that inflammation results in neoplastic transformation by enhancing abnormal proliferation of the crypt epithelium. The monoclonal antibody Ki-67 recognises an antigen present in the nuclei of cells in the proliferative phases of the cell cycle and is used as a marker of prognosis in colorectal cancer (Li et al., 2015).

Tumour cells also need to acquire the ability to escape from programmed cell death mediated by apoptosis (Strasser et al., 2011). Abnormalities in apoptotic function is an essential for the pathogenesis of colorectal cancer. Survivin (Birc5) is a member of the inhibitor of apoptosis protein (IAP) family and is highly upregulated in colon cancer (Hernandez et al., 2011, Adamkov et al., 2015). We decided to probe the protein Survivin in the colonic tumours to evaluate the upregulation of IAP proteins.

The increased distance between the tumour cell and blood vessels reduces the chance of getting oxygen and thus, decreases the survival of tumour cells. Therefore, neoangiogenesis in tumour microenvironment is vital to provide sufficient oxygen and nutrients to tumour cell survival and growth and positively correlates with advanced tumour stage (Cao et al., 2009). Vascular endothelial growth factor (VEGF) is a potent angiogenic factor that contributes to the new blood vessel network. Therefore, we looked at the expression of Ki-67, VEGF and Survivin biomarkers to investigate cell proliferation, angiogenesis, and anti-apoptotic activity of the Winnie x *Nlrp3*$^{-/-}$.

To investigate potential mechanisms resulting in tumorigenesis in Winnie x *Nlrp3*$^{-/-}$, we quantified transcription of a panel of genes associated with colorectal neoplasms. A limitation of the use of RNA from total tissues in microarray gene expression analysis is that the results are reflective of the whole colon tissue and does not specify exact cellular types. However, it avoids the modification of gene expression taking place during cell purification procedure, and it has been shown to be able to provide important information regarding the main molecular pathways affected in different experimental or pathological conditions.

To analyse the molecular mechanism involved in Winnie x *Nlrp3*$^{-/-}$ tumorigenesis we investigated prominent pathways upregulated in CAC. The phosphatidylinositol 3-kinase (PI3K)–Akt pathway and its downstream factors were examined first, as they are directly involved in proliferation and growth in epithelial cells and is associated in colitis associated colorectal cancer (Sheng et al., 2003, Danielsen et al., 2015). Activation of PI3K/Akt signalling has been shown to inhibit Glycogen synthase 3 kinase β (GSK3β) through phosphorylation at Ser9 (Huber et al., 2005).

Another major pathway involved with proliferation and differentiation in colon cancer is the over activated Wnt/β-catenin signalling pathway (Pandurangan et al., 2018, Lapham et al., 2009). GSK3β is an important negative regulator of Wnt/β-catenin signalling pathway. In the absence of Wnt signalling, Axin, GSK3β and APC assemble as a complex to phosphorylate β-catenin for ubiquitin mediated degradation (Wu and Pan, 2010). In the presence of Wnt signalling, the inactivated form P-GSK3β protects β-catenin from degradation and the stabilized β-catenin in the cytoplasm translocates into the nucleus and activates transcription factors TCF/ lymphocyte enhancer factor (TCF/LEF), and stimulates the expression of target genes such as *c-Myc, c-Jun, CD44* and *Cyclin-D1* (Lapham et al., 2009, Van der Flier et al., 2007, Dang, 2012, Shtutman et al., 1999).

The protooncogene *c-Myc* regulates the expression of genes involved in cell proliferation, DNA repair, apoptosis and angiogenesis (Luscher, 2012, Gabay et al., 2014). High expression of c-myc is seen in colon tissue of colon cancer patients (Lee et al., 2016). c-myc is required for the transcription of many cell cycle related proto-oncogenes such as *Cyclin D1* (Obaya et al., 1999) which is overexpressed in colon cancer (Wang et al., 1998).

Several studies have established that breakdown of the homeostasis by dysbiosis of gut microbiota results in the development of various pathological conditions such as IBD and

CAC (Nistal et al., 2015, Tjalsma et al., 2012, Xavier and Podolsky, 2007). Concomitantly other studies have shown association of pro-carcinogenic microbiota with tumour tissues (Kostic et al., 2012, Sanapareddy et al., 2012). Tjalsma et al proposed a model where bacteria with the tumorigenic potential act as drivers and induce inflammation, which facilitates the expansion of tumorigenic bacterial strains. Another study showed that that intestinal inflammation in the IL-10 knock out mouse model modifies gut microbial communities and promotes the growth of genotoxic bacteria (Arthur et al., 2012).

Another way that gut microorganisms contribute to IBD and CAC development is by their role in inducing pro-inflammatory responses, such as the induction of IL-17 or IFN γ pro-inflammatory responses (Ivanov et al., 2009, Kim et al., 2007, Kullberg et al., 1998) or specifically activate suppressive IL-10 cytokine response (Mazmanian et al., 2008, Round and Mazmanian, 2009).

The NLRP3 inflammasome is a well characterised gut innate immune sensor belonging to the NLR family that are critical for modulating microbial ecology to protect against tumour development (Kamada et al., 2013, Allen et al., 2010). In the study we have generated a CAC mouse model in the absence of NLRP3 and the analysis of the microbiota will define the protective role of NLRP3 inflammasome in CAC penetrance.

One of the mechanisms by which gut microbiota contributes to tumorigenesis is by changing the production of metabolite levels. Gut microbiota produces short-chain fatty acids (SCFAs) acetate, propionate, and butyrate by fermentation of dietary fibre (den Besten et al., 2013). Specifically, the phylum Bacteroidetes members mainly produce acetate and propionate, whereas the phylum Firmicutes produce butyrate as its primary metabolic end product (Koh et al., 2016). Among SCFAs, butyrate has received most attention for its effects on colonic health (Hamer et al., 2008). Butyrate has been suggested to be protective against colon cancer due to its antiproliferative effects in most human colon cancer cell lines and its ability to increase apoptosis and differentiation (Hague et al., 1993, Heerdt et al., 1994).

From the studies mentioned above, to understand the pathogenesis of CAC it is vital to identify the involvement of unique microbiota and metabolomic composition in colon cancer. Therefore, in the study, we have performed an in-depth analysis of the microbiota and its metabolomics in Winnie x *Nlrp3*$^{-/-}$ CAC model. We analysed Winnie x *Nlrp3*$^{-/-}$ at

two age groups, 12 and 16 weeks to identify dynamic changes in the composition of microbiota and metabolomics during disease progression.

6.2 Methodology

Confocal Immunofluorescence Microscopy

Sections were deparaffinized and subjected to 0.01 mol/L citrate buffer (C9999, Sigma-Aldrich) at pH 6.0 antigen retrieval at 121^0C for 4 minutes in a decloaking chamber (DC2012, Biocare Medical). Nonspecific binding was blocked by a 1-hour incubation in blocking buffer (0.1 mol/L phosphate-buffered saline/5% normal goat serum/0.05% Tween-20) at room temperature in the dark. Immunofluorescence staining was performed using specific antibodies against VEGF using Anti-VEGF antibody (ab2350, Abcam) and Ki67 with anti-Ki67 antibody (ab15580, Abcam**)** in the dark, at room temperature overnight at 4^0C. Sections then were incubated for 1 hour in the dark with appropriate Alexa Fluor–conjugated secondary antibody (ab150077, Abcam). Section were incubated with Hoechst 33342 (62249, Thermo Fisher Scientific) diluted in PBS (P3813, Sigma-Aldrich) and mounted with ProLong Gold Antifade (P36930, ThermoFisher Scientific). Slides were examined using a Laser Scanning Confocal Inverted Microscope (FV1200, Olympus).

RNA extraction and RT-PCR

Colonic tissue was homogenised using an Omni Mixer Homogenizer (3410B05, Thomas Scientific) and RNA extracted using the RNeasy Mini spin column kit (74104, Qiagen) according to the manufacturer's instructions. Integrity and concentration of extracted RNA was assessed using Eppendorf Biophotometer (D30, Eppendorf). Complementary DNA (cDNA) was synthesised from RNA samples using the iScript gDNA clear cDNA synthesis kit (1725034, Bio-Rad) using reaction conditions suggested by the manufacturer. 100 ng of cDNA from each sample was added to a PCR reaction including TaqMan Fast Advanced Master Mix (4444557, Applied Biosystems) and a single gene-specific TaqMan probe/primer set. *Aim2* (Assay ID: Mm01295719_m1, Thermo Fisher Scientific), *Nlrc3* (Assay ID: Mm00615968_m1, Thermo Fisher Scientific), *Nlrc4* (Assay ID: Mm01239561_m1, Thermo Fisher Scientific), *Nlrp6* (Assay ID: Mm00460229_m1, Thermo Fisher Scientific), *Bcl2* (Assay ID: Mm00437796_m1, Thermo Fisher Scientific) and *Sod2* (Assay ID: Mm01313000_m1, Thermo Fisher Scientific), *Pparγ* (Assay ID: Mm00440940_m1, Thermo Fisher Scientific), *Myc* (Assay ID: Mm00487804_m1, Thermo

Fisher Scientific), *Survivin* (Assay ID: Mm00599749_m1, Thermo Fisher Scientific) and *Cdk2* (Assay ID: Mm00486279_m1, Thermo Fisher Scientific). Thermal cycling was performed using a StepOnePlus Real-Time PCR Systems (4376600, Applied Biosystems). Gene expression was quantified using the comparative (ΔΔCT) method where the threshold cycle (CT) for each gene was normalised to reference gene Gapdh (Assay ID: Mm99999915_g1, Thermo Fisher Scientific). Relative gene expression was presented as $2^{-\Delta CT}$.

Colorectal neoplasms PrimerPCR assay

Colonic tissue was homogenised using Omni Mixer Homogenizer (3410B05, Thomas Scientific) and RNA extracted using the RNeasy Mini spin column kit (74104, Qiagen) according to the manufacturer's instructions. Integrity and concentration of extracted RNA was assessed using Eppendorf Biophotometer (D30, Eppendorf). Complementary DNA (cDNA) was synthesised from RNA samples using the iScript gDNA clear cDNA synthesis kit (1725034, Bio-Rad) using reaction conditions suggested by the manufacturer. 20 ng of cDNA of samples were loaded into the Colorectal neoplasms Tier 1 M96 plate (10035050, Bio-Rad). The mRNA levels of 88 genes involved in Colorectal neoplasms were detected at the same time using SYBR Green Supermix (1708880, Bio-Rad) on StepOnePlus Real-Time PCR Systems (4376600, Applied Biosystems). The data were analyzed by using Bio-Rad PrimePCR Analysis software.

Western blotting

Proteins from the colon were extracted using RIPA lysis buffer (R0278 Sigma) supplemented with proteinase and phosphatase inhibitors (4693116001, Sigma-Aldrich). The protein samples were resolved on 4-20% mini-protean precast SDS-PAGE gels (4561093, Biorad) and transferred onto polyvinylidene difluoride membrane (1620177, Biorad) using transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3) in a wet-transfer system. Membranes were blocked in 5% (wt/vol) dried milk in TBS-T (50 mM Tris/HCL, pH 7.6, 150 mM NaCl and 0.1% (vol/vol) Tween-20) for 1 hour at room temperature. Membranes were incubated with primary antibody diluted in 5% (wt/vol) dried milk in TBS-T and then with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody diluted in 5% (wt/vol) dried milk in TBS-T for 1 hour. Membranes were developed using SuperSignal West Pico chemiluminescent substrate

(34580, Thermo Fisher Scientific). Membranes were stripped using Restore PLUS Western blot stripping buffer (46430, Thermo Fisher Scientific) before being re-probed.

Primary antibodies used were Rabbit GSK-3β (12456, Cell Signalling Technology), Phospho Anti-beta Catenin (9561T, Cell Signalling Technology), CD44 Mouse mAb (3570, Cell Signalling Technology) Cyclin D1 Rabbit (2978, Cell Signalling Technology) LEF1 Rabbit mAb (2230, Cell Signalling Technology) c-Myc Rabbit mAb (5605, Cell Signalling Technology) TCF1/TCF7 Rabbit mAb (2203, Cell Signalling Technology) at a concentration of 1 in 1,000; Anti-Ki67 antibody (ab16667, Abcam), Anti-VEGF antibody (ab2350, Abcam), Anti-PI 3 Kinase (Ab182651, Abcam), Anti-AKT1 antibody (ab81283, Abcam), IL-1β (NB600-633, Novus Biologicals), β-actin (4967, Cell Signalling Technology) at 1:500. Secondary HRP-conjugated antibodies used were, anti-rabbit IgG (7076, Cell Signalling Technology) and Anti-mouse IgG (7076, Cell Signalling Technology) at 1:2000.

Faecal and Mucosal Sample Collection and Preparation

Faecal samples were collected from separately housed mice in a manner similar to Langille et al. (Langille et al., 2014). To minimize contamination, the mice were on the day of sampling mice had no access to food and water, and sterile forceps were used for faecal sample collection. At least 2 pellets (100–150 mg of the sample) were collected from each mouse and immediately transferred into a sterile microcentrifuge tube, stored at -80°C for 16S rRNA gene sequencing.

Mucosal samples were collected on the exact age after the mice were euthanized by CO2 asphyxiation. Colon was excised, and luminal contents of colonic-mucosa were carefully collected with sterile forceps immediately transferred into a sterile microcentrifuge tube. These samples were immediately stored at -80°C for subsequent metabolomic analysis.

Microbiota analysis using 16s rRNA High-Throughput sequencing

The total DNA was extracted from faecal samples using the QIAamp DNA Stool Mini Kit (51504, Qiagen). The samples underwent high-throughput sequencing on the Illumina MiSeq platform at the Australian Genome Research Facility (University of Queensland, Brisbane, QLD, Australia). PCR amplicons spanning the 16S rRNA V3-V4 hypervariable region with 27F forward Primer (5'-AGAGTTTGATCMTGGCTCAG-3') and 519R reverse Primer (5'-GWATTACCGCGGCKGCTG-3') were sequenced. Paired-end reads

were assembled by aligning the forward and reverse reads using PEAR1 (version 0.9.5). Primers were identified and trimmed. Trimmed sequences were processed using Quantitative Insights into Microbial Ecology (QIIME 1.8) 4 USEARCH 2.3 (version 8.0.1623) and UPARSE software (Edgar, 2013). Using USEARCH tools, sequences were quality filtered; full-length duplicate sequences were removed and sorted by abundance. Singletons or unique reads in the data set were discarded. Sequences were clustered followed by chimera filtering using "rdp_gold" database as a reference (Edgar et al., 2011). To obtain several reads in each Operational taxonomic units (OTUs), reads were mapped back to OTUs with a minimum identity of 97%. Using QIIME, taxonomy was assigned using Greengenes database5 (Version 13_8, Aug 2013) (Kuczynski et al., 2012). Image analysis was performed in real time by the MiSeq Control Software (MCS) v2.6.2.1 and Real-Time Analysis (RTA) v1.18.54, running on the instrument computer. RTA performs real-time base calling on the MiSeq instrument computer. Then the Illumina bcl2fastq 2.20.0.422 pipeline was used to generate the sequence data (Edgar et al., 2011, Kuczynski et al., 2012). 16S rRNA gene sequences were analysed using MEGAN6 (Community edition version) (Huson and Mitra, 2012), Microbiome analyst (Dhariwal et al., 2017) and QIIME. Statistical analysis of Brady-Curtis dissimilarities was calculated using the relative abundances of bacterial genera was conducted using Adonis function in R (version 3.2).

Metabolomics analysis for SCFA

All samples were prepared and derivatised following the protocol developed by Furuhashi et al., (2018) (Furuhashi et al., 2018), with some modifications. Briefly, mucosal samples (stored at -80°C) were weighed to ±0.1 mg accuracy. These samples (100 – 150 mg fresh weight) were added to a sterile 1.5 mL bead-beating tube (NAVYR5, Next Advance). Isobutanol (78-83-1, Merck) (10% in water, volume = 1.0 mL) was added to each sample, followed by two 30 second, 4,000 rpm homogenization pulses sandwiched between a 20-second pause interval with a Precellys Evolution Homogenizer (Bertin Instruments). The samples were subsequently centrifuged at 15,700 g for 6 minutes.

The supernatant (675 µL) was transferred to a clean round bottomed 2 mL centrifuge tube (0030120094, Eppendorf,) and 20 mM, 125 µL of NaOH (30620, Merck) and 400 µL of chloroform (102445, Merck) were added. The samples were briefly vortexed and centrifuged at 15,700 rpm for 3 minutes. The aqueous phase (upper layer, 400 µL) was transferred to a new clean round bottomed 2 ml centrifuge tube (0030120094, Eppendorf)

containing a boiling chip (1.07913, Sigma-Aldrich). 100µL Pyridine (109728, Merck), 80 µL isobutanol (78-83-1, Merck) and 70 µL milliQ water were added and the samples were subjected to gentle vortexing followed by addition of 50 µL isobutyl chloroformate (177989, Sigma-Aldrich). The tube was then opened to release any generated gases and was allowed to stand for about 1 minute. 150 µL Hexane (675393, Sigma-Aldrich) was then added to each tube, which was capped and vortexed prior to centrifugation at 15,700 g for 4 minutes. The upper phase (100 µL) was subsequently transferred to GC autosampler vials fitted with salinized glass inserts; Malathion (36143, Sigma-Aldrich) (1 µL, equivalent to 2.5 µg/mL) was added as an internal standard.

The GC-MS analysis was performed on an Agilent 6890B gas chromatograph (GC) oven coupled to a 5977B mass spectrometer (MS) detector (Agilent Technologies, Mulgrave, VIC, Australia) fitted with an MPS autosampler (Gerstel GmbH & Co.KG, Deutschland, Germany). The GC oven was fitted with two 15 m HP-5MS columns (0.25 mm ID and 0.25 µm film thickness; 19091S-431 UI, Agilent Technologies, Mulgrave, VIC, Australia) coupled to each other through a purged ultimate union (PUU) for the use of post-run backflushing. The sample (1.0 µL) was introduced via a multimode inlet (MMI) operated in split mode (1:20). The column was maintained at 40°C for 5 min, followed by an increase to 250°C at a rate of 10°C/min. This was followed by a second increment to 310°C at a rate of 60°C/min. The column was held at 310°C for 1 min. The mass spectrometer was kept in Extractor ion mode (EI mode) at 70 eV. The GC-MS ion source temperature and transfer line were kept at 250°C and 280°C, respectively. Detector voltage was kept at 1054 V. The MS detector was turned off for the first 3 min and, at 4.0 – 4.8 min and 12.5 – 13.2-min time windows until the excess derivatization reagent and chloroformate/hexane solvents were eluted from the column. This ensured that the source filament was not saturated and damaged. The scan range was kept in the range of *m/z* 35 – 350 (35 – 350 Daltons). Data acquisition and spectral analysis were performed as described previously (Vemuri et al., 2018) and qualitative identification of metabolites was performed according to the Metabolomics Standard Initiative (MSI) chemical analysis workgroup (Sansone et al., 2007) using standard GC-MS reference metabolite libraries (NIST 17, Agilent Fiehn RTL Library [G166766A, Agilent Technologies] with the use of Kovats retention indices based on a reference n-alkane standard (C8-C40 Alkanes Calibration Standard, Sigma-Aldrich).

Faecal Metabolomics

The samples were subjected to derivatisation to increase volatility before subjecting to GC-MS analysis. Briefly, faecal samples (n = 5, weight = 40 mg) were freeze-dried and suspended in 1 mL methanol (322415, Sigma-Aldrich), supplemented with 10 µg/mL adonitol (A5502, Sigma-Aldrich) as an internal standard in sterile 2 mL bead-beating tube. The samples were homogenized by bead beating for 30 s and then centrifuged at 570 g/ 4°C for 15 minutes. The supernatant (50 µL) was transferred to a fresh centrifuge tube (1.5 mL) and dried in a vacuum evaporator centrifuge (LabGear, Brisbane, QLD, Australia) at 35°C. Methoxyamine-HCl (20 mg/mL in Pyridine) (226904, Sigma-Aldrich) was added (40 µL) and samples were incubated at 30°C/ 1400 rpm (ThermoMixer C, Eppendorf) for 90 minutes. This was followed by slylation with 70 µL BSTFA (33027, Sigma-Aldrich) at 37°C/ 1400 rpm for 30 minutes. Pre-derivatized 13C-Stearic acid (10 µg/mL) was added (1 µL) as the QA/QC internal standard. The mixture was briefly vortexed and centrifuged at 15,700 g for 5 min. The aliquot was transferred to vials for GC-MS analysis.

The GC-MS analysis was performed on an Agilent 6890B gas chromatograph (GC) oven coupled to a 5977B mass spectrometer (MS) detector (Agilent Technologies, Mulgrave, VIC, Australia) fitted with an MPS autosampler (Gerstel GmbH & Co.KG, Deutschland, Germany). The GC-MS conditions were as stated previously (Beale et al., 2013, Karpe et al., 2015, Beale et al., 2014). Data acquisition and spectral analysis were performed using the Qualitative Analysis software (Version B.08.00) of MassHunter workstation. Qualitative identification of the compounds was performed according to the Metabolomics Standard Initiative (MSI) chemical analysis workgroup (Members et al., 2007) using standard GC-MS reference metabolite libraries (NIST 17, Fiehn Metabolomics RTL Library [G166766A, Agilent Technologies] and the Golm database) and with the use of Kovats retention indices based on a reference n-alkane standard (C8-C40 Alkanes Calibration Standard, Sigma-Aldrich). For peak integration, a 5- point detection filtering (default settings) was set with a start threshold of 0.2 and a stop threshold of 0.0 for 10 scans per sample. Procedural blanks (n = 7) were analyzed randomly throughout the sequence batch. The obtained data was processed on Quantitative analysis platform of MassHunter workstation and exported as a Microsoft Excel output file for statistical analysis.

Data Analysis and Multivariate Analysis

GC-MS data imported to Microsoft Excel platform was normalized with respect to the internal standard adonitol (relative standard deviation = 11.257%). The normalized data was further log-transformed and auto-scaled (mean-centered) before statistical analysis (French et al., 2018). To determine overall microbial variation in four groups, we used a principal coordinate analysis (PCoA), Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram, Neighbour-Net (a distance-based method for constructing phylogenetic networks) hierarchical clustering with Brady-Curtis ecological indexing and Euclidean distances as the similarity measure and Ward's linkage as clustering algorithm (Yu et al., 2017, French et al., 2018). For analysis of metabolome variations, Principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA) and orthogonal (O) PLS-DA were used. Because PLS-DA can overfit data, we used 1000 permutations to validate these models. The OPLS-DA was used to identify discrimination between metabolites contributing to classification.

Statistical Analysis

Graph Pad Prism version 7.0 for Windows was used for the statistical analysis. The data were analyzed using the Wilcoxon Mann–Whitney Test (multiple comparisons), with $p < 0.05$ set as the level of statistical significance. For microbial comparative analysis, a linear discriminant effect size (LEfSe) analysis was performed ($\alpha = 0.05$), logarithmic Linear Discriminant Analysis (LDA) score threshold = 1.0. A MetaboAnalyst (Version 4.0) data annotation approach and Kyoto Encyclopaedia of Genes and Genomes (KEGG) Pathway Database were used for the hierarchical clustering analysis, significance analysis for microarrays (SAM) along with the variable importance of projection (VIP) (Sun et al., 2013). The SAM and VIP methods are a well-established statistical method for metabolites, was used to select the most discriminant and interesting biomarkers (Hayakawa et al., 2016).

6.3 Results

6.3.1 Analysis of proliferation, apoptosis and angiogenesis in colonic tissue

Since colonic tumorigenesis is associated with abnormal proliferation of the crypt epithelium, we assessed the localisation of the Ki-67 marker of proliferating cells. We observed that colonic mucosal epithelial cell proliferation, as indicated by Ki-67 staining,

was decreased in Winnie mice compared with Winnie x *Nlrp3*$^{-/-}$ (Figure 6.1a). In contrast, the proliferative signal intensity of the colonic crypt of Winnie x *Nlrp3*$^{-/-}$ at week 16 was more intense than Winnie x *Nlrp3*$^{-/-}$ at week 12 suggesting increased proliferation of the colonic epithelium.

Tumour cells develop mechanisms to avoid programmed cell death mediated by apoptosis by upregulating IAPs such as Survivin. We looked in to the expression of Survivin in colonic tissue of Winnie and Winnie x *Nlrp3*$^{-/-}$. We found an elevated expression of VEGF in Winnie x *Nlrp3*$^{-/-}$ when compare to Winnie mice (Figure 6.1b).

Since neoangiogenesis is essential for the tumour growth, we evaluated the expression of potent angiogenic factor VEGF in colonic tissue of Winnie and Winnie x *Nlrp3*$^{-/-}$. We found an elevated expression of VEGF in Winnie x *Nlrp3*$^{-/-}$ when compare to Winnie mice (Figure 6.1c).

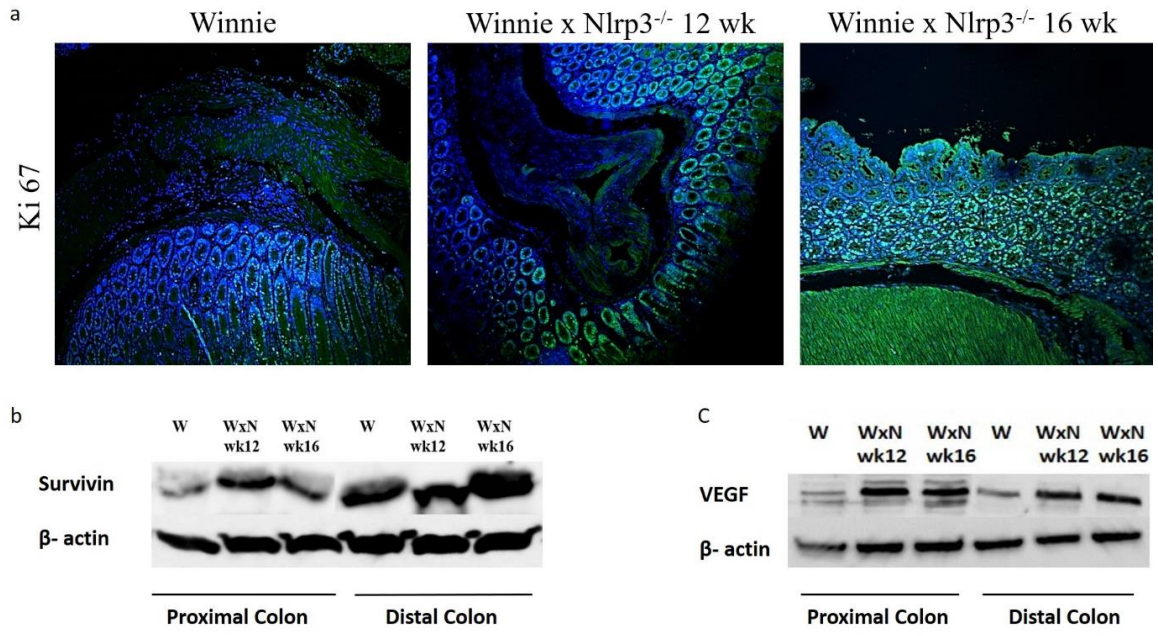


Figure 6.1: Expression of Ki67, Survivin and VEGF. Representative immunofluorescence confocal images showing expression of Ki-67 in colonic tissue in Winnie (W), Winnie x *Nlrp3*$^{-/-}$ 12-week (WxN 12wk) and Winnie x *Nlrp3*$^{-/-}$ 16-week (WxN 16wk) mice proximal and distal colon tissue. Western blotting showing expression levels of (b) Survivin (c) VEGF (the loading control used was β-actin) in Winnie (W), Winnie x *Nlrp3*$^{-/-}$ 12-week (WxN 12wk) and Winnie x *Nlrp3*$^{-/-}$ 16-week (WxN 16wk) mice proximal and distal colon tissue. Data are from one experiment representative of three independent experiments.

6.3.2 Analysis of Inflammasome mRNA expression profile in colonic tissue

Quantitative reverse-transcriptase PCR analysis revealed a significant reduction in the expression of the gene encoding NLRP6 in Winnie x *Nlrp3*$^{-/-}$ at week 12 tumour tissue of the proximal colon when compared with non-tumour-associated tissue in the proximal colon of C57BL/6 (Figure 6.5a). In the distal colon tissue of Winnie x *Nlrp3*$^{-/-}$ week 12 a significant reduction of the expression of AIM2, NLRC4 and NLRP6 were observed (Figure 6.5b). In Winnie x *Nlrp3*$^{-/-}$ week 16 proximal and distal colon tissue expressed a significantly increased expression of AIM2, NLRC3, NLRC4 and NLRP6 when compare to C57BL/6 expression (Figure 6.5b).

6.3.3 Analysis of colorectal cancer biomarker array in colonic tissue

In this study, we used Bio-Rad PrimePCR™ microarray gene expression platform for analysis of altered molecular signatures of colorectal neoplasia in the mouse colon of Winnie x *Nlrp3*$^{-/-}$ 12 week and 16 weeks relative to Winnie. We have presented the dysregulated biomarkers significant in relative expression as cluster gram and tabled the relative P-values in Figure 6.3.

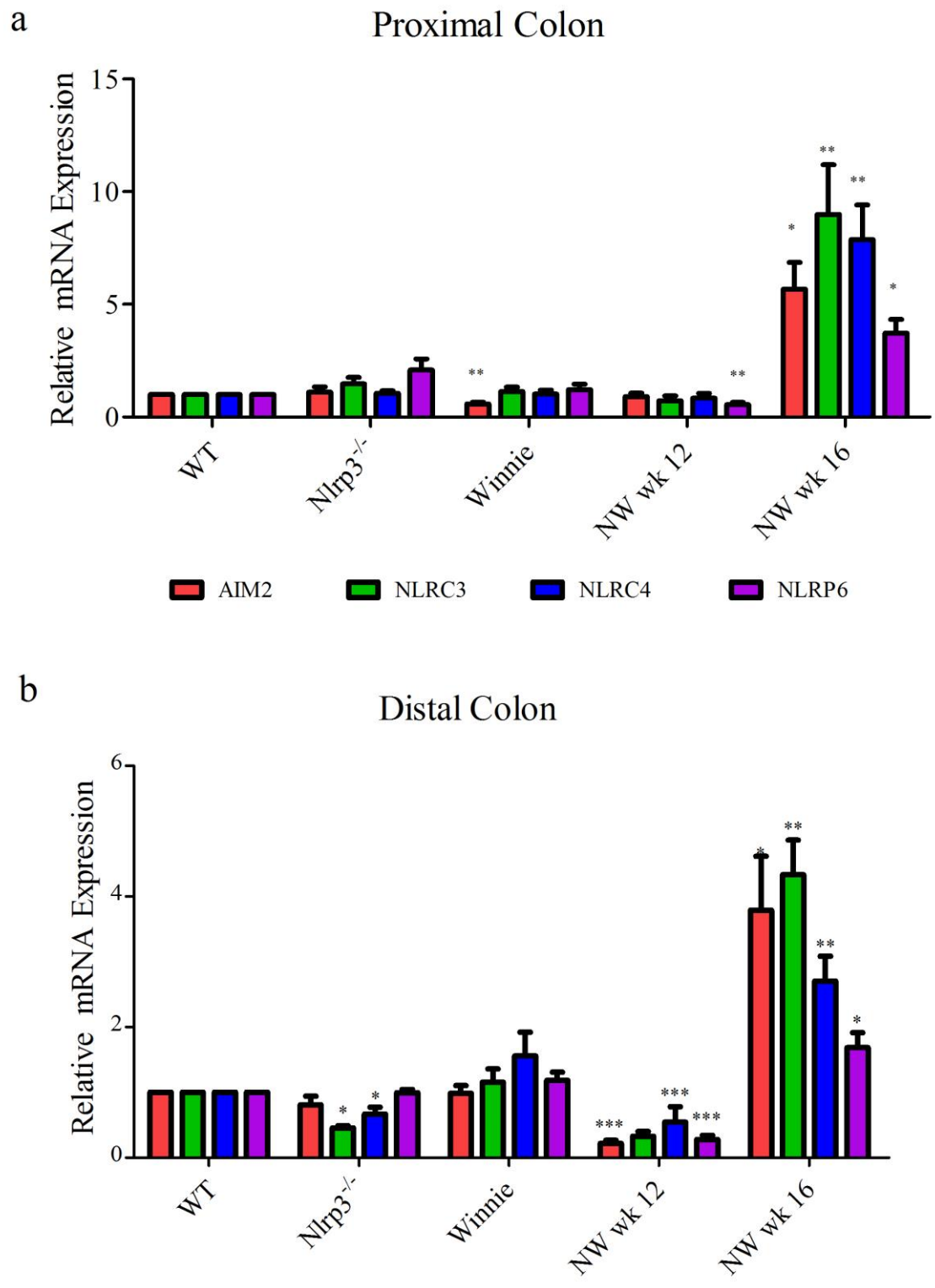
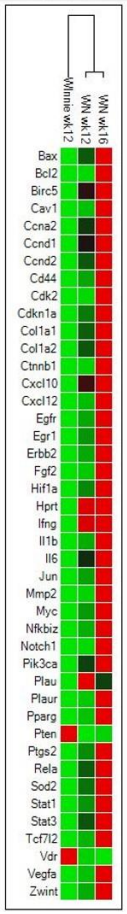


Figure 6.2: The mRNA expressions of AIM2, NLRC3, NLRC4 and NLRP6 in (a) proximal and (b) distal colon tissue were determined by real time PCR. *Nlrp3*$^{-/-}$, Winnie, Winnie x *Nlrp3*$^{-/-}$ data was normalised to the C57BL/6 samples. Data are expressed as the mean±SEM (n=6 per group) *P < 0.05, **P < 0.01, ***P < 0.001 (one sample t test).

a

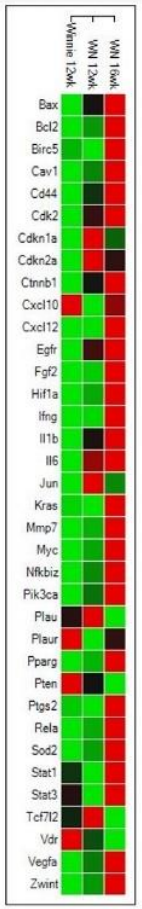


Target	Sample	Relative Normalized Expression	Compared to Regulation Threshold	P-Value
Bax	WN wk12	71.30810	Up regulated	0.025715
Bax	WN wk16	246.39705	Up regulated	0.006620
Bcl2	WN wk12	42.26772	Up regulated	0.197545
Bcl2	WN wk16	2004.63390	Up regulated	0.134164
Birc5	WN wk12	342.93567	Up regulated	0.000048
Birc5	WN wk16	589.31336	Up regulated	0.010506
Cav1	WN wk12	5.82739	Up regulated	0.025476
Cav1	WN wk16	79.68176	Up regulated	0.085931
Ccnd1	WN wk12	19.32675	Up regulated	0.098079
Ccnd1	WN wk16	35.40478	Up regulated	0.000003
Ccnd2	WN wk12	23.94652	Up regulated	0.065021
Ccnd2	WN wk16	79.14033	Up regulated	0.260554
Cd44	WN wk12	48.36639	Up regulated	0.117064
Cd44	WN wk16	429.21387	Up regulated	0.157639
Cdk2	WN wk12	65.08685	Up regulated	0.011027
Cdk2	WN wk16	3004.15004	Up regulated	0.064576
Cdkn1a	WN wk12	34.93604	Up regulated	0.110270
Cdkn1a	WN wk16	166.05198	Up regulated	0.107901
Col1a2	WN wk12	49.51501	Up regulated	0.013054
Col1a2	WN wk16	163.16538	Up regulated	0.143421
Ctnnb1	WN wk12	68.52148	Up regulated	0.142616
Ctnnb1	WN wk16	2048.86744	Up regulated	0.040040
Cxcl10	WN wk12	7.21654	Up regulated	0.072322
Cxcl10	WN wk16	10.79800	Up regulated	0.022302

Target	Sample	Relative Normalized Expression	Compared to Regulation Threshold	P-Value
Cxcl12	WN wk12	10.41708	Up regulated	0.026451
Cxcl12	WN wk16	131.78352	Up regulated	0.105528
Egfr	WN wk12	10.43518	Up regulated	0.054129
Egfr	WN wk16	87.70513	Up regulated	0.000110
Egr1	WN wk12	57.23733	Up regulated	0.091804
Egr1	WN wk16	378.65809	Up regulated	0.016626
Fgf2	WN wk12	30.12292	Up regulated	0.252230
Fgf2	WN wk16	617.48356	Up regulated	0.014582
Hif1a	WN wk12	23.41434	Up regulated	0.014648
Hif1a	WN wk16	129.02217	Up regulated	0.127943
Hprt	WN wk12	1.51547	No change	0.367652
Hprt	WN wk16	1.52322	No change	0.349265
Il1b	WN wk12	9.96857	Up regulated	0.034284
Il1b	WN wk16	90.37732	Up regulated	0.093667
Il6	WN wk12	303.38705	Up regulated	0.190239
Il6	WN wk16	723.11020	Up regulated	0.000411
Jun	WN wk12	76.96419	Up regulated	0.023591
Jun	WN wk16	632.42008	Up regulated	0.000000
Myc	WN wk12	84.30806	Up regulated	0.017320
Myc	WN wk16	630.61806	Up regulated	0.022311
Nfkbiz	WN wk12	90.25540	Up regulated	0.000074
Nfkbiz	WN wk16	976.85245	Up regulated	0.123186
Pik3ca	WN wk12	23.36776	Up regulated	0.014349
Pik3ca	WN wk16	64.16525	Up regulated	0.000083

Target	Sample	Relative Normalized Expression	Compared to Regulation Threshold	P-Value
Plaur	WN wk12	446.30432	Up regulated	0.001505
Plaur	WN wk16	9799.91480	Up regulated	0.050340
Pparg	WN wk12	66.12622	Up regulated	0.016473
Pparg	WN wk16	1106.69927	Up regulated	0.079832
Pten	WN wk12	0.04648	Down regulated	0.010459
Pten	WN wk16	0.07460	Down regulated	0.000083
Ptgs2	WN wk12	8.98449	Up regulated	0.002503
Ptgs2	WN wk16	49.57922	Up regulated	0.010393
Rela	WN wk12	21.37043	Up regulated	0.150755
Rela	WN wk16	72.82143	Up regulated	0.089920
Sod2	WN wk12	1273.77349	Up regulated	0.006114
Sod2	WN wk16	5674.77648	Up regulated	0.057158
Stat1	WN wk12	7.47284	Up regulated	0.016279
Stat1	WN wk16	44.46982	Up regulated	0.037790
Stat3	WN wk12	19.16203	Up regulated	0.025710
Stat3	WN wk16	61.74318	Up regulated	0.103831
Tcf7l2	WN wk12	83.36929	Up regulated	0.054760
Tcf7l2	WN wk16	1007.65856	Up regulated	0.029934
Vdr	WN wk12	0.05880	Down regulated	0.011245
Vdr	WN wk16	0.02654	Down regulated	0.010459
Vegfa	WN wk12	8.32468	Up regulated	0.002247
Vegfa	WN wk16	122.18834	Up regulated	0.045787
Zwint	WN wk12	45.25022	Up regulated	0.047793
Zwint	WN wk16	445.52033	Up regulated	0.048472

b



Target	Sample	Relative Normalized Expression	Compared to Regulation Threshold	P-Value
Bax	WN wk12	1.13586	No change	0.768296
Bax	WN wk16	1.26585	No change	0.686266
Bcl2	WN wk12	3.94418	No change	0.360012
Bcl2	WN wk16	22.78455	Up regulated	0.190230
Birc5	WN wk12	0.73626	No change	0.552134
Birc5	WN wk16	4.02700	Up regulated	0.047552
Cav1	WN wk12	3.71052	No change	0.347305
Cav1	WN wk16	16.20117	Up regulated	0.101661
Ccnd1	WN wk12	4.46198	Up regulated	0.429136
Ccnd1	WN wk16	9.70041	Up regulated	0.151005
Ccnd2	WN wk12	4.28696	Up regulated	0.005111
Ccnd2	WN wk16	3.00214	No change	0.358499
Cd44	WN wk12	1.74399	No change	0.189596
Cd44	WN wk16	1.19570	No change	0.705834
Cdk2	WN wk12	4.06330	Up regulated	0.428572
Cdk2	WN wk16	2.78996	No change	0.067536
Cdkn1a	WN wk12	0.83802	No change	0.642491
Cdkn1a	WN wk16	2.24066	No change	0.303669
Col1a2	WN wk12	1.26684	No change	0.674725
Col1a2	WN wk16	0.91288	No change	0.949190
Ctnnb1	WN wk12	4.48417	Up regulated	0.287764
Ctnnb1	WN wk16	8.11484	Up regulated	0.020061
Cxcl10	WN wk12	0.45737	No change	0.805642
Cxcl10	WN wk16	0.91584	No change	0.684384

Target	Sample	Relative Normalized Expression	Compared to Regulation Threshold	P-Value
Cxcl12	WN wk12	1.01470	No change	0.915629
Cxcl12	WN wk16	2.84551	No change	0.131934
Egfr	WN wk12	1.39758	No change	0.392255
Egfr	WN wk16	1.61346	No change	0.298447
Egr1	WN wk12	1.54300	No change	0.494986
Egr1	WN wk16	2.57812	No change	0.263183
Fgf2	WN wk12	1.31330	No change	0.138103
Fgf2	WN wk16	8.84179	Up regulated	0.231366
Hif1a	WN wk12	4.85400	Up regulated	0.396409
Hif1a	WN wk16	38.51808	Up regulated	0.086565
Hprt	WN wk12	1.06863	No change	0.803976
Hprt	WN wk16	4.50310	Up regulated	0.126020
Il1b	WN wk12	2.67300	No change	0.357648
Il1b	WN wk16	4.15570	Up regulated	0.323030
Il6	WN wk12	2.40778	No change	0.441378
Il6	WN wk16	2.64458	No change	0.223077
Jun	WN wk12	2.82916	No change	0.429416
Jun	WN wk16	1.31068	No change	0.605993
Myc	WN wk12	2.84594	No change	0.430518
Myc	WN wk16	17.56301	Up regulated	0.290445
Nfkbiz	WN wk12	1.86688	No change	0.594051
Nfkbiz	WN wk16	6.23921	Up regulated	0.120361
Pik3ca	WN wk12	4.39200	Up regulated	0.388940
Pik3ca	WN wk16	15.84834	Up regulated	0.141944

Target	Sample	Relative Normalized Expression	Compared to Regulation Threshold	P-Value
Plaur	WN wk12	0.60866	No change	0.899641
Plaur	WN wk16	0.84202	No change	0.672496
Pparg	WN wk12	39.26037	Up regulated	0.419659
Pparg	WN wk16	472.71123	Up regulated	0.053900
Pten	WN wk12	0.00005	Down regulated	0.002288
Pten	WN wk16	0.50141	No change	0.831843
Ptgs2	WN wk12	4.36824	Up regulated	0.003861
Ptgs2	WN wk16	0.84716	No change	0.979845
Rela	WN wk12	3.21159	No change	0.382991
Rela	WN wk16	23.43520	Up regulated	0.131526
Sod2	WN wk12	7.80574	Up regulated	0.421251
Sod2	WN wk16	58.52667	Up regulated	0.075385
Stat1	WN wk12	0.79209	No change	0.724739
Stat1	WN wk16	1.31590	No change	0.407109
Stat3	WN wk12	0.94139	No change	0.873298
Stat3	WN wk16	1.04677	No change	0.885043
Tcf7l2	WN wk12	1.50591	No change	0.521190
Tcf7l2	WN wk16	0.59626	No change	0.381166
Vdr	WN wk12	0.51789	No change	0.512939
Vdr	WN wk16	0.29264	No change	0.148350
Vegfa	WN wk12	2.30415	No change	0.430301
Vegfa	WN wk16	7.46851	Up regulated	0.087043
Zwint	WN wk12	9.73140	Up regulated	0.005788
Zwint	WN wk16	2.20485	No change	0.490136

Figure 6.3: The expression of mRNA of Colorectal neoplasm biomarkers in Winnie and Winnie x *Nlrp3*$^{-/-}$ at 12 week and 16week (a) proximal and (b) distal colon tissue were determined by Bio-Rad PrimePCR^{TM}. The cluster gram and relative normal expression of Winnie x *Nlrp3*$^{-/-}$ at 12 week and 16-week data was normalised to the C57BL/6 samples by the Bio-Rad PrimePCR Analysis software. Data are expressed as the mean±SEM (n=2 per group) P values obtained by one sample t test.

The data shows significant upregulation of *Bcl2, Birc5 (Survivin), Cav1, Cd44, Hif1a, Myc, Pparγ, Stat3, Ptgs2 (Cox2), Sod2, Vegfa* and *Zwint* in Winnie x *Nlrp3*$^{-/-}$ when compared to Winnie. We observed a significant down regulation of the genes *Pten* and *Vdr* in Winnie x *Nlrp3*$^{-/-}$ when compared to Winnie. These dysregulated biomarkers could hint at molecular pathways in colonic carcinogenesis in Winnie x *Nlrp3*$^{-/-}$.

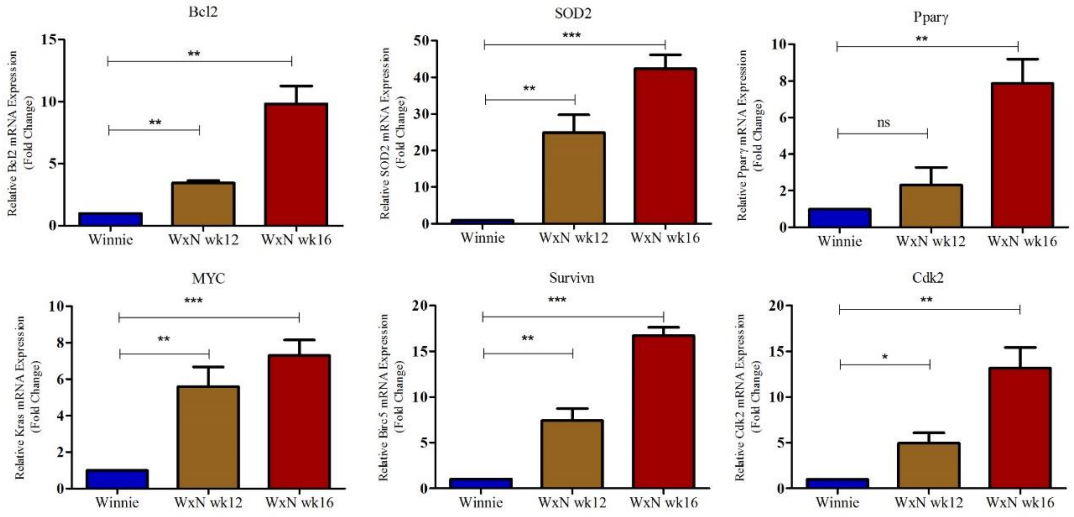


Figure 6.4: The mRNA expressions of *Bcl2, Sod2, Pparγ,Myc, Survivin* and *Cdk2* in distal colon tissue were determined by real time PCR. Winnie x *Nlrp3*$^{-/-}$ 12-week (WxN wk12) and Winnie x *Nlrp3*$^{-/-}$ 16-week (WxN wk16) were normalised to the C57BL/6 samples. Data are expressed as the mean±SEM (n=6 per group) *P < 0.05, **P < 0.01, ***P < 0.001 (one sample t test).

Using RT-PCR, we validated the upregulated expression of six genes significantly involved in many biologic processes relevant to cancer such as apoptosis, cell proliferation, and cell cycle arrest. All these genes showed a significant upregulation in relative expression in the distal colon of Winnie x *Nlrp3*$^{-/-}$ 12-week and Winnie x *Nlrp3*$^{-/-}$ 16-week compared to Winnie (Figure 6.4).

6.3.4 Analysis of upregulated molecular signalling pathways

To examine whether PI3k/AKT pathway is upregulated in the Winnie x *Nlrp3*$^{-/-}$, we first investigated the activity of PI3K and saw that PI3K phosphorylation increased in Winnie x *Nlrp3*$^{-/-}$ in comparison to Winnie colon tissue (Figure 6.5j). Then we analysed the phosphorylation of Akt at Ser473, which is strongly dependent upon PI3K activity. Figure 6.5i shows that Winnie x *Nlrp3*$^{-/-}$ clearly resulted in a progressive and significant increase in Akt phosphorylation.

We analysed the protein expression profile of the Wnt/β-catenin signalling pathway in the novel model Winnie x *Nlrp3*$^{-/-}$ colonic tissue. Firstly, we analysed the phosphorylation status of GSK-3β and observed upregulation in Winnie x *Nlrp3*$^{-/-}$ colonic tissue (Figure 6.5g). We then monitored the status of phosphorylated β-catenin, the downstream substrate of GSK-3β, and showed a significant decrease in this protein (Figure 6.5f). Next, we monitored the expression status of LEF1 and TCF and observed an increased expression in Winnie x *Nlrp3*$^{-/-}$ colonic tissue (Figure 6.5 d and e). We then analysed c-myc (Figure 6.5 c), Cyclin D1(Figure 6.5b) and CD44 (Figure 6.5c) the chief genes upregulated by transcription factor TCF/LEF. CD44 showed an increased expression in Winnie x *Nlrp3*$^{-/-}$ 12 week and 16week (Figure 6.5a) while c-myc and were only expressed in the Winnie x *Nlrp3*$^{-/-}$ distal colonic tissue at 16 weeks. We analysed the IL-1β expression and found it increased in Winnie x *Nlrp3*$^{-/-}$ colonic tissue (Figure 6.5h)

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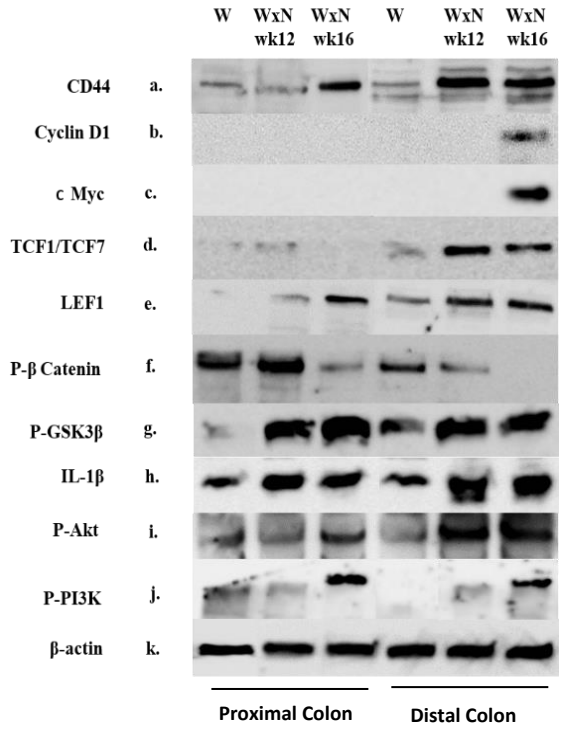


Figure 6.5: Western blotting of upregulated upstream signaling molecules within the PI3K/Akt and Wnt/β-catenin pathway in Winnie, Winnie x *Nlrp3*$^{-/-}$ 12-week (WN 12wk) and Winnie x *Nlrp3*$^{-/-}$ 16-week (WN 16wk) mice Proximal and Distal colon tissue. Data are from one experiment representative of three independent experiments.

6.3.5 Analysis of Microbiota

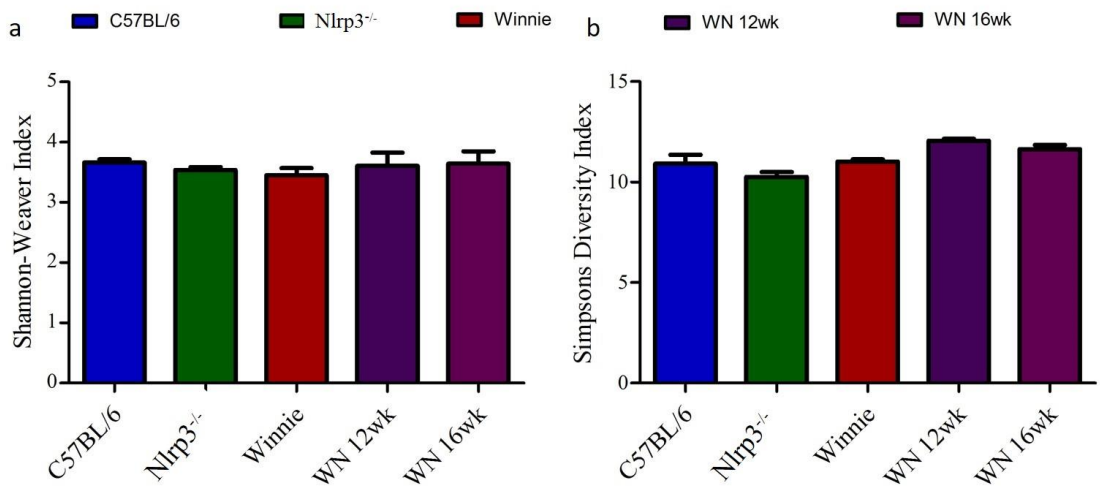
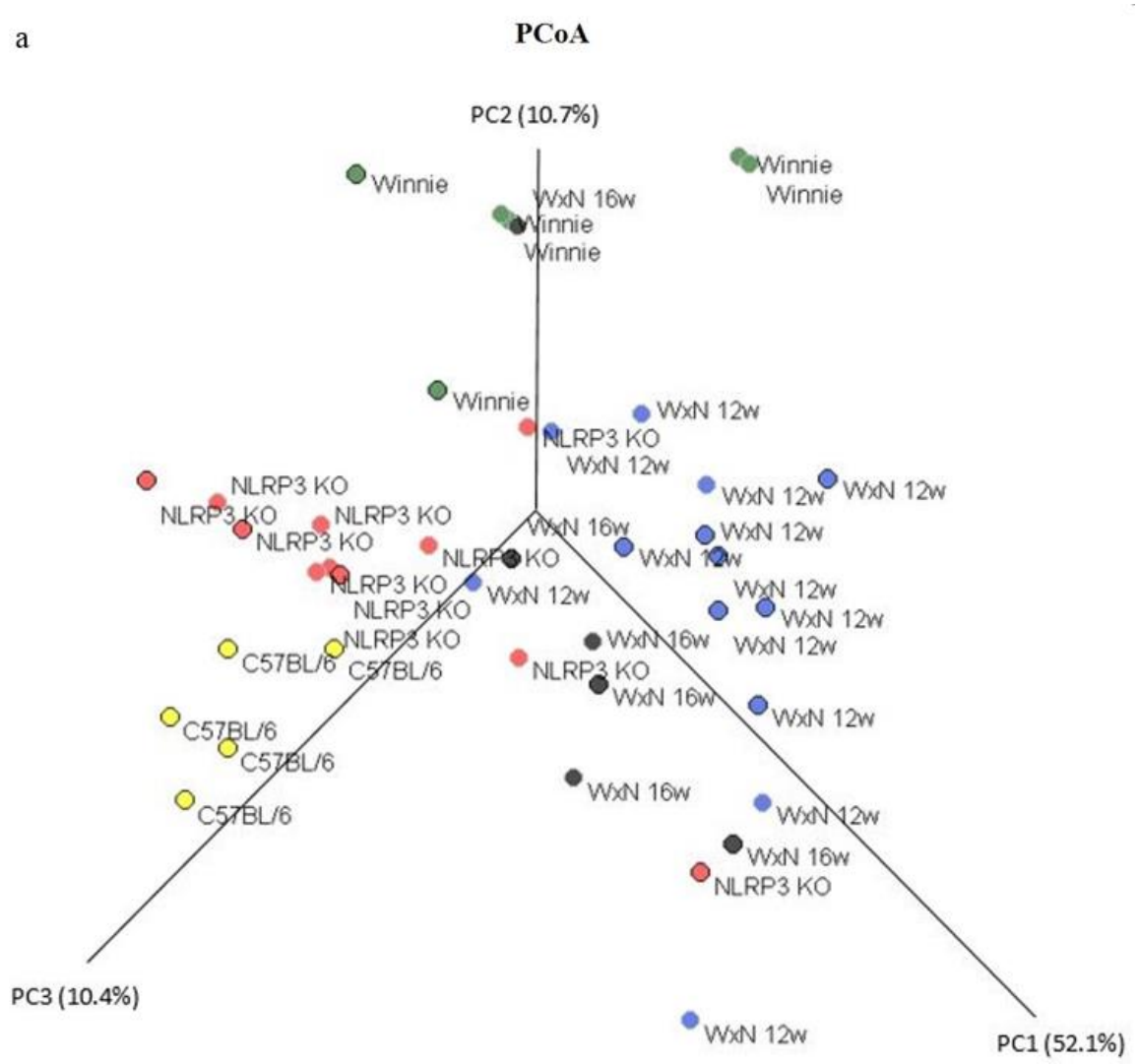


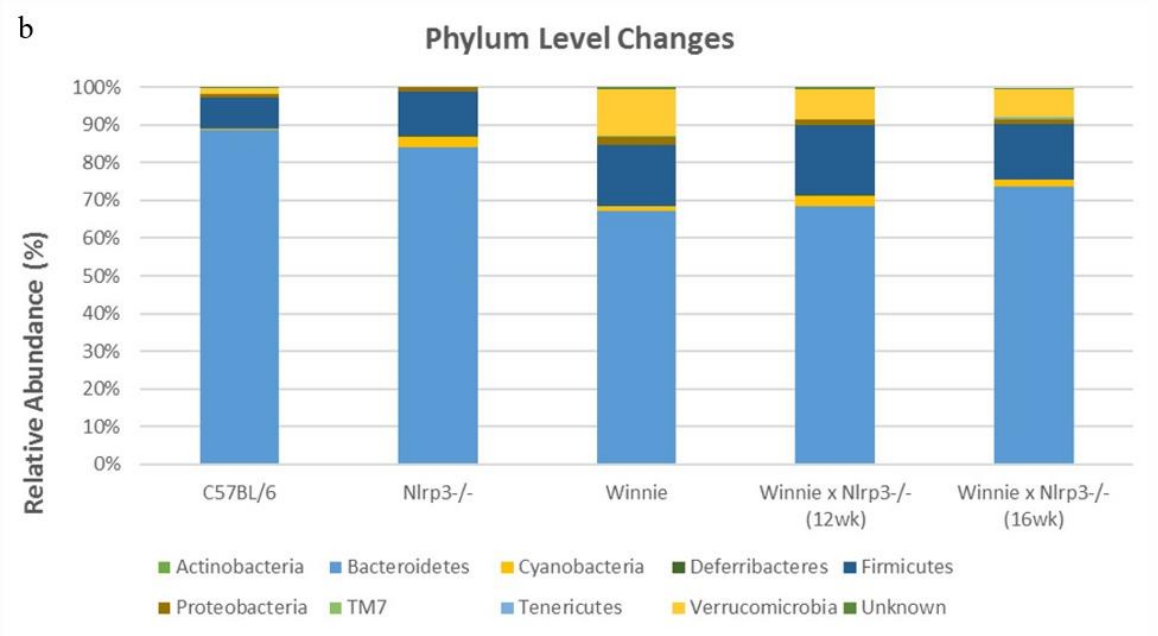
Figure 6.6: C57BL/6, *Nlrp3*$^{-/-}$, Winnie, Winnie x *Nlrp3*$^{-/-}$ 12-week (WN 12wk) and Winnie x *Nlrp3*$^{-/-}$ 16-week (WN 16wk) mice faecal bacteria 16S rRNA V3-V4 region was sequenced with Miseq technology (Illumina) and Shannon-Weaver index (a) and Simpson Diversity index (b) were compared between the mouse groups. Data are shown as means ± SEM (n=3).

To investigate whether an altered gut microbiota directly underlies the pathogenesis of colorectal cancer, we analysed the levels of major bacterial species of gut microbiota in stool samples of separately housed (single housed) C57BL/6, *Nlrp3*$^{-/-}$, Winnie and Winnie x *Nlrp3*$^{-/-}$ mouse groups in three independent experiments. Faecal microbiota profiling was performed using 16S rRNA gene sequencing-based method.

We calculated the Shannon and Simpsons index which are diversity indexes encompassing comprehensive OTU richness and OTU evenness, and the larger the index, the more abundant the species in the samples. We found that the richness and diversity of the intestinal microbiota do not differ among the groups as seen by the Shannon-Weaver and Simpsons indexes (Figure 6.6).

Interestingly in our study, the PCoA plots of phylogeny with Brady-Curtis ecological indexing using ward clustering showed a clear separation of each group with four distinct clusters at the OTU level among 4 groups (Figure 6.7a). Interestingly, PCoA plot showed the control groups (C57BL/6, *Nlrp3*$^{-/-}$) were concentrated in one quadrant, while the remaining diseased groups (Winnie x *Nlrp3*$^{-/-}$, Winnie) were concentrated in another quadrant, this indicates that the overall structure of the gut microbiota shows a striking difference in the composition of microbial communities between the control and diseased models.


a
PCoA
PC2 (10.7%)
PC3 (10.4%)
PC1 (52.1%)
Winnie
WxN 16w
NLRP3 KO
WxN 12w
C57BL/6


b
Phylum Level Changes
Relative Abundance (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
C57BL/6
Nlrp3-/-
Winnie
Winnie x Nlrp3-/- (12wk)
Winnie x Nlrp3-/- (16wk)
Actinobacteria
Bacteroidetes
Cyanobacteria
Deferribacteres
Firmicutes
Proteobacteria
TM7
Tenericutes
Verrucomicrobia
Unknown

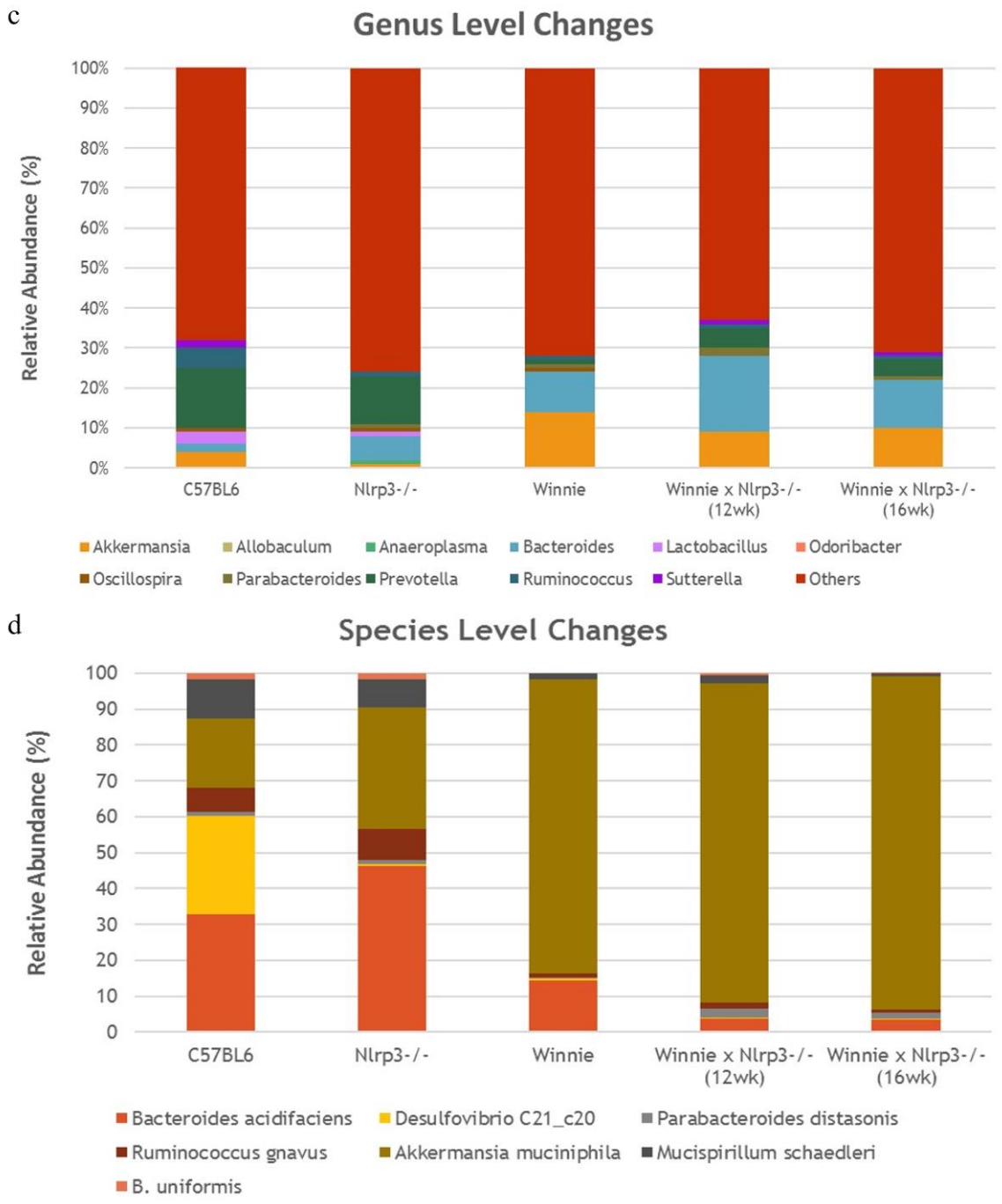


Figure 6.7: The gut microbiota changes observed in C57BL/6, *Nlrp3*$^{-/-}$, Winnie, Winnie x *Nlrp3*$^{-/-}$ 12 week (WN 12wk) and Winnie x *Nlrp3*$^{-/-}$ 16 week (WN 16wk) were differentiated by principle coordinate analysis (PCoA) using Brady Curtis ecological index distance method (a). The gut microbiota composition profiles at phylum (b), genus levels (C) and species level (d) in all 5 groups revealed by 16S rRNA gene sequencing.

We classified around 99% of the total microbial abundance into nine major phyla in all groups, the rest were allocated as unknown (Figure 6.7b). At the phylum level, the abundance of dominant intestinal bacteria such as *Bacteroidetes* becomes significantly reduced ($P < 0.05$) in the Winnie and Winnie x *Nlrp3*$^{-/-}$ genotypes while the *Firmicutes*

have increased when compared to C57BL/6. In line with the findings a study has shown a decrease in overall abundance of *Bacteroidetes* in IBD (Blumberg and Powrie, 2012). We found a significantly higher ($P < 0.05$) abundance in *Verrucomicrobia* in Winnie and Winnie x *Nlrp3*$^{-/-}$ genotypes when compare to C57BL/6.

At the genus level, the distribution of microbial populations of Winnie and Winnie x *Nlrp3*$^{-/-}$ was markedly different when compared to C57BL/6 group. The experimental genotypes were enriched with higher populations of colitogenic *Akkermansia* and *Bacteroides* while reducing the abundances of beneficial *Prevotella* and *Lactobacillus* as shown in Figure 6.7 c.

At the species level, there was a significant increase ($p < 0.05$) in *Akkermansia munciphilia* levels, and significant decrease ($p < 0.05$) in *Desulfovibrio C21_c20*, *Ruminococcus gnavus* and *Bacteroides acidifaciences* levels in the Winnie and Winnie x *Nlrp3*$^{-/-}$, when compared to C57BL/6 group (Figure 6.7 d).

6.3.6 Analysis of Metabolomics and SCFA

To gain an untargeted overview of CAC related changes in dominant gut metabolites, we analysed mucosal samples using a GC-MS platform. A total of 68 metabolites of different functional groups such as sugars, amino acids and biogenic amines were detected. An unsupervised PCA was used to obtain an overview of the samples, and all samples were clearly discriminated, as shown in Figure 6.8a. PCA analysis showed four distinct clustering of the four genotypes. The samples from control groups (C57BL/6 and *Nlrp3*$^{-/-}$) were different from Winnie and Winnie x *Nlrp3*$^{-/-}$ disease groups. The control and disease groups formed two clusters that were different and showed great divergence compared to the control groups. Similarly, the *Nlrp3*$^{-/-}$ group formed a cluster which was different from the Winnie group and more closely related to the WT group. The biplot for PC1 and PC2 showed the compounds with the greatest impact on the division among the samples, as shown in Figure 6.8b. In order to provide a better visualization and to carry out the class information of each variable, a supervised PLS-DA approach was used to evaluate the metabolic patterns of Winnie group and Winnie x *Nlrp3*$^{-/-}$ group (Figure 6.8C). The accuracy, R2X, R2Y and Q2 of PLS-DA score analysis was 0.875, 0.821, and 0.517, respectively (Figure 6.8C), indicating the classification was well suited for the models, and the genotypes were classified clearly. This analysis indicated the clear differences in the

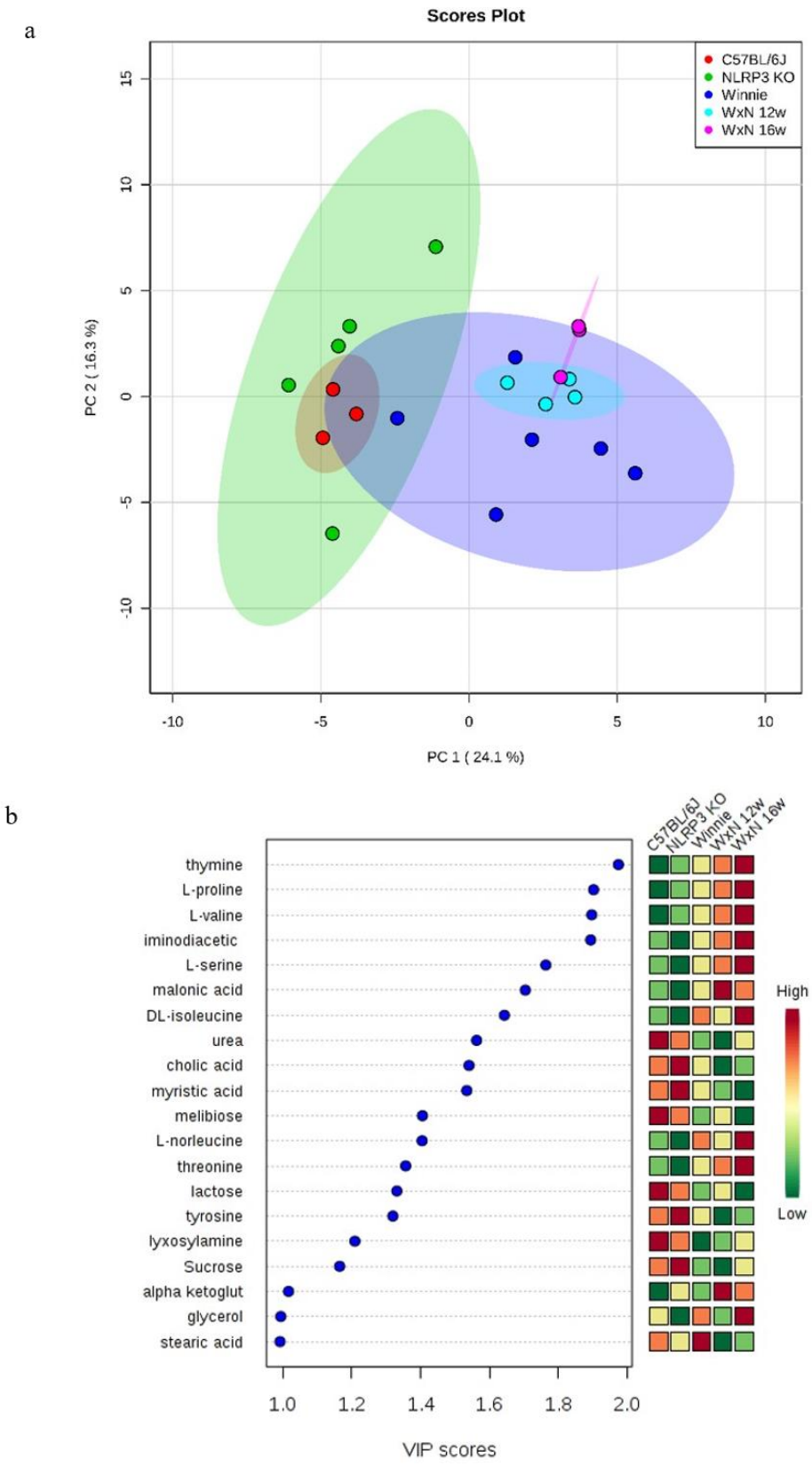


Figure 6.8: a. Principle component analysis (PCA) plot of mucosal collected from C57BL/6, *Nlrp3*$^{-/-}$, Winnie, Winnie x *Nlrp3*$^{-/-}$ 12 week (WN 12wk) and Winnie x *Nlrp3*$^{-/-}$ 16 week (WN 16wk) showing divergence. b. Key compounds separating all four groups based on variable importance in projection (VIP) score plot.

metabolic profiles of mice in Winnie group and Winnie x *Nlrp3*$^{-/-}$ group, which suggests that CAC induced significant biochemical changes.

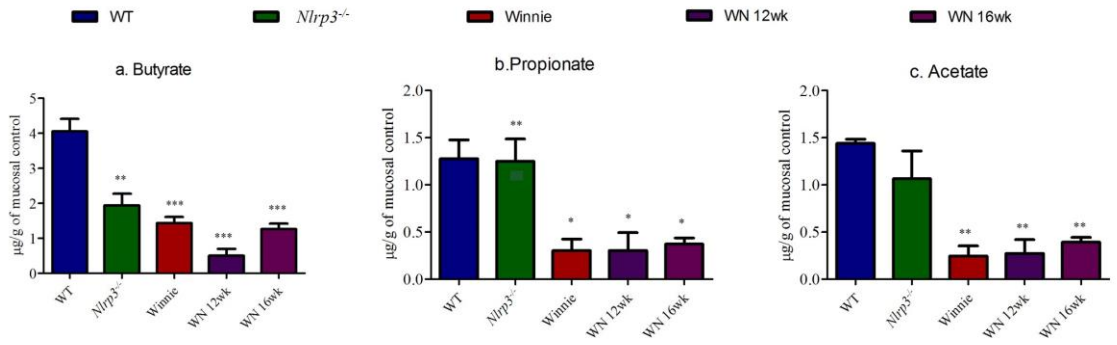


Figure 6.9: Short chain fatty acid (SCFA) distribution observed in C57BL/6, *Nlrp3*$^{-/-}$, Winnie, Winnie x *Nlrp3*$^{-/-}$ 12 week (WN 12wk) and Winnie x *Nlrp3*$^{-/-}$ 16 week (WN 16wk). The concentrations of butyrate (a), propionate (b) and acetate (c) were measured by GC-MS analysis from mucosal contents. *P < 0.05, **P<0.01, ***P<0.001 (One-way ANOVA with Tukey's post-hoc test and data are expressed as the mean ± SEM).

A total of 15 volatile compounds were identified in the untargeted metabolomics from mucosal samples in all four groups Table 6.1. These 15 metabolites included the three major SCFA of butyrate, propionate, and acetate. C57BL/6 and *Nlrp3*$^{-/-}$ did not show any difference in the production of butyrate, propionate, and acetate in the mucosal samples. Notably, in the mucosal samples, butyrate, propionate, and acetate levels were significantly decreased in both Winnie and Winnie x *Nlrp3*$^{-/-}$ groups compared to the control groups.

Table 6.1: List of volatile fatty acids (VFA) identified in C57BL/6, *Nlrp3*$^{-/-}$, Winnie, Winnie x *Nlrp3*$^{-/-}$ 12wk, Winnie x *Nlrp3*$^{-/-}$ 16wk n=3. The concentrations of VFA in the mucosal content (samples were measured by GC-MS analysis from mucosal contents at the end of the study. *P < 0.05 (One-way ANOVA with Tukey's post-hoc test and data are expressed as the mean ± SEM).

Volatile fatty acids	C57BL/6	*Nlrp3*$^{-/-}$	Winnie	WN 12wk	WN 16wk
Isobutyl acetate	1.441 ± 0.045 **	0.74 ± 0.11 ***	0.25 ± 0.11 ***	0.18 ± 0.05 ***	0.33 ± 0.11 ***
Isobutyl ether	1.49 ± 0.24 **	0.47 ± 0.06 ***	0.25 ± 0.02 ***	0.28 ± 0.09 ***	0.40 ± 0.07 ***
d-Proline, N-methoxycarbonyl-, heptyl ester	0.75 ± 0.26	0.47 ± 0.14 **	0.29 ± 0.04 **	0.19 ± 0.04 *	0.31 ± 0.06
2-Propen-1-ol, 2-methyl	0.75 ± 0.03	0.47 ± 0.14 **	0.29 ± 0.04 **	0.19 ± 0.04 *	0.31 ± 0.06
Carbonic acid	3.2 ± 0.16	2.91 ± 0.8	1.3 ± 0.22	1.06 ± 0.37 *	1.6 ± 0.35
Ethanedioic acid	1.725 ± 0.1709	0.33 ± 0.02 ***	0.22 ± 0.04 ***	0.08 ± 0.04 ***	0.16 ± 0.06 ***
Butane, 1,1-dibutoxy	0.61 ± 0.24	0.16 ± 0.02	0.087 ± 0.017 *	0.04 ± 0.005 *	0.08 ± 0.01 *
D-Alanine, N-allyloxycarbonyl-, nonyl ester	0.73 ± 0.06	0.39 ± 0.05 **	0.22 ± 0.04 ***	0.18 ± 0.03 ***	0.2 ± 0.03 ***
L-Isoleucine, N-isopropyl-, methyl ester	0.39 ± 0.02	0.246 ± 0.03 **	0.16 ± 0.02 ***	0.06 ± 0.003***	0.1 ± 0.009 ***
Propanedioic acid, methyl-, dibutyl ester	0.008 ± 0.001	0.006 ± 0.0003	0.003 ± 0.0005	0.003 ± 0.0008	0.015±0.004
Succinic acid, 3-chlorophenyl 4-methoxybenzyl ester	0.25 ± 0.07	0.12 ± 0.02	0.01 ± 0.0028 **	0.003 ± 0.001**	0.008 ± 0.002**
d-Proline, N-isobutoxycarbonyl-, isobutyl ester	0.09 ± 0.002	0.08 ± 0.004	0.10 ± 0.08	0.015 ± 0.006	0.06 ± 0.03
Carbonic acid, monoamide, N-propyl-N-(hept-2-yl)-, butyl ester	0.02 ± 0.003	0.02 ± 0.01	0.004 ± 0.001	0.004 ± 0.002	0.007 ± 0.003
l-Norvaline, N-isobutoxycarbonyl-, isobutyl ester	0.05 ± 0.003 *	0.03 ± 0.009 ***	0.004 ± 0.001 ***	0.004 ± 0.002 ***	0.007 ± 0.003 *
Benzeneacetic acid, 2-methylpropyl ester	0.023 ± 0.01	0.03 ± 0.01	0.006 ± 0.003	0.01 ± 0.001	0.02 ±0.006

6.4 Discussion

Initiation of CAC occurs when activated inflammatory signalling pathways (Grivennikov, 2013) lead to aberrant intestinal epithelial and disruption of homeostasis. The epithelial cells undergo abnormal proliferation with decreased differentiation and apoptosis leading to early dysplasia and adenomatous polyps. High expression of Ki-67, a cell proliferation marker, is considered an independent prognostic marker in colorectal cancer (Melling et al., 2016). The results revealed a significant increase in numbers of both Ki-67^{+} cells in intestinal crypt of Winnie x *Nlrp3*$^{-/-}$ compared to Winnie mice confirming colitis associated carcinogenesis in colonic tissue.

Survivin is a member of the inhibitor of apoptosis (IAP) family that counteracts cell death and controls mitotic progression and is overexpressed in colorectal cancer (Li et al., 1999, Altieri, 2003). Evidence from cDNA microarray demonstrated that *Survivin* also plays an important role in the pathogenesis of CRC (Williams et al., 2003, Huang et al., 2013). Similarly, the results demonstrated an upregulation at the protein and the gene level for *Survivin* in the colonic tissue of Winnie x *Nlrp3*$^{-/-}$ compared to Winnie mice.

Kim et al has shown that *Survivin* is a new target gene of the TCF/ catenin signalling axis, coupling increased cell proliferation to enhanced cell survival in intestinal crypt cells (Kim et al., 2003). Similarly, other studies have shown compelling evidence that the presence of *Survivin* in CRC is strongly associated with the expression of Bcl2 and ki-67 demonstrating that Survivin expression is related to CRC proliferation (Danilewicz et al., 2015, Cai et al., 2015). Likewise, the results show a significant correlation between the increased expression of Survivin, Bcl2 and ki-67.

In colon cancer VEGF expression is elevated in tumour tissue and positively correlates with advanced tumour stage as well as reduced life expectancy (Cao et al., 2009, Bendardaf et al., 2008). CAC mouse model (AOM/DSS-treated) has shown elevated expression of VEGF with anti-VEGF treatment reducing the tumour growth (Waldner et al., 2010a). In another study inhibition of VEGF dramatically reduced the tumour development, angiogenesis and cell proliferation (Waldner et al., 2010b). Moreover, it has been shown that β-catenin/Wnt signalling regulates VEGF expression in CRC (Easwaran et al., 2003). In addition to tumorigenesis, angiogenesis and VEGF is associated in the pathogenesis of

inflammatory bowel disease (Costa et al., 2007). In line with these studies the results show VEGF expression in both Winnie and Winnie x *Nlrp3*$^{-/-}$ mouse models.

Absent in melanoma 2 (AIM2) is a pyrin-HIN protein that binds cytosolic double-stranded DNA and forms the active inflammasome complex with the common inflammasome adaptor ASC (Hornung et al., 2009, Fernandes-Alnemri et al., 2009). A previous study showed that AIM2 gene reduction or absence is observed in several cancer types (Patsos et al., 2010).

A study has shown that AIM2 physically interacted with and limited activation of DNA-dependent protein kinase (DNA-PK) (Merkle et al., 2002). DNA-PK is a PI3K-related family member that promotes Akt phosphorylation (Feng et al., 2004, Li et al., 2013, Lu et al., 2006). A recent study has shown AIM2 reduced Akt activation and tumor burden in CAC mouse models. The same study showed the effects of AIM2 on CAC were independent of inflammasome activation and IL-1β and were primarily mediated by a non–bone marrow source of AIM2 (Wilson et al., 2015). Similarly, in the study we have observed a significant reduction of AIM2 in the tumour tissue of the distal colon of the Winnie x *Nlrp3*$^{-/-}$ mice at 12 weeks while the expression of IL-1β cytokine was significantly high.

However, we observed a significant increase in expression of the inflammasomes AIM2, NLRC3, NLRC4 and NLRP6 in Winnie x *Nlrp3*$^{-/-}$ week 16 mouse colons. At 16 weeks Winnie x *Nlrp3*$^{-/-}$ mice have solid tumours with invasive carcinomas with high expression of ROS and DNA damage markers. Overproduction of these DAMPS could lead to the over activation of inflammasomes and could explain the upregulation of inflammasomes genes seen in Winnie x *Nlrp3*$^{-/-}$ week 16.

The results of the quantified transcription of genes associated with colorectal neoplasms revealed significant upregulation of many genes related to multiple signalling pathways. These target genes are involved in tumour cell survival (e.g. *Bcl2, Survivin*), proliferation (e.g. *cdk2, Myc, cd44, jun*), angiogenesis (e.g. *Hif1alpha, Vegfa*), inflammation (e.g. *IL-6, Sod2, Cox2*) and epithelial mesenchymal transition (EMT) (e.g. *zwint*).

Bcl2 overexpression is known to suppress caspase-1 mediated cell death (Miura et al.,1993). Similarly, we saw a significant upregulation of *Bcl2* in Winnie x *Nlrp3*$^{-/-}$ colon which could lead to the survival of malignant cells initiating tumorigenesis.

Cdk2 is cyclin-dependent kinase 2 is a promoter of cell cycle and mitosis and is associated with colon cancer (Yamamoto et al., 1998). qPCR analysis showed a significant increase of *Cdk2* in Winnie x *Nlrp3*$^{-/-}$ colon which could aid in the aberrant cell proliferation observed in the colonic epithelium of Winnie x *Nlrp3*$^{-/-}$.

Myc plays a fundamental role in several cellular functions, including regulation of cell growth, proliferation, metabolism, differentiation, apoptosis, and angiogenesis (Gabay et al., 2014). We found an increase in *Myc* expression in the Winnie x *Nlrp3*$^{-/-}$ distal colon at the protein and gene level indicating *Myc* plays a prominent role in tumorigenesis in the CAC model.

Ptgs2 (*Cox*-2) plays an important role in invasion, angiogenesis and metastasis of colorectal carcinogenesis. Elevated expression of *Ptgs2* is found to be exhibited in 85% of colorectal tumours (Gupta and Dubois, 2001) and later upregulated with the advance stages (Castellone et al., 2005). It is expressed early by IECs in response to growth factors and proinflammatory cytokines during inflammation (Ishikawa and Herschman, 2010) and plays a very crucial role in prognosis of CRC at an early stage (Umar et al., 2004). *Cox-2* may promote tumour development through its ability to induce the expression of antiapoptotic proteins such as *Bcl-2* and result in resistance to apoptosis. In addition, overexpression of *Cox-2* is associated with elevated levels of MMPs and increased migration of malignant cells (Gupta and Dubois, 2001). Similarly, we observed a significant upregulation of *Cox-2* gene expression in Winnie x *Nlrp3*$^{-/-}$ colon indicating this molecule plays an essential role in the tumorigenesis of the novel mouse model.

Interestingly, the results showed a significant decrease in the gene expression of *Vdr* (vitamin D receptor) in the Winnie x *Nlrp3*$^{-/-}$ colon. *Vdr* mediates the active metabolite of Vitamin D to produce varieties of biological effects (Baker et al., 1988). Some studies have shown that *Vdr* mRNA and protein levels are reduced in the early stages of colon cancer (Giardina et al., 2015, Anderson et al., 2006). Vitamin D has been proposed as a chemotherapeutic agent against cancer but the loss of *Vdr* reduce the beneficial effects of high vitamin D status on cancer progression (Fleet et al., 2012). The above studies speculate an association between the reduction of *Vdr* and colon cancer corroborating our findings.

Pparγ is a metabolic regulator of lipid anabolism. Activation of *Pparδ* can induce *Cox-2* expression via a self-amplifying loop contributing to colorectal carcinogenesis(Oshio et al., 2008). Likewise, the Winnie x *Nlrp3*$^{-/-}$ colon revealed a significant upregulation of *Pparγ.*

We observed an upregulation of *Stat3* in the Winnie x *Nlrp3*$^{-/-}$ colon. Activation of *Stat3*, by proinflammatory cytokines leads to the regulation of anti-apoptotic genes such as *Bcl-2*, cell cycle regulators, like *Cyclin D1* or *c-Myc* and angiogenic factors, such as and VEGF (Grivennikov and Karin, 2010a, Yu et al., 2009). Indeed, inactivation of *Stat3* in intestinal epithelium affects both cell survival and cell proliferation during acute or chronic colitis (Bollrath et al., 2009, Grivennikov et al., 2009, Pickert et al., 2009) and in cancer model decreases CAC tumor multiplicity and diminishes tumor growth (Bollrath et al., 2009, Grivennikov et al., 2009).

In addition, we observed a significant increase in the gene expression of *Hif1a* in the Winnie x *Nlrp3*$^{-/-}$ colon. *Hif1a* is a master regulator of cell response to hypoxia by activating genes involved in angiogenesis, apoptosis, and energy metabolism (Bakker et al., 2007, Shi et al., 2013).

Interestingly, we observed a high expression of EMT-associated transcription factor *Zwint* in the Winnie x *Nlrp3*$^{-/-}$ colon. *Zwint* is upregulated in the early event of invasion and metastasis and is regulated by the Wnt/ β -catenin signalling pathway (Rodriguez-Salas et al., 2017, Abbas and Dutta, 2009).

Several inflammatory pathways, including of NF-κB, PI3K/Akt pathways can drive β-catenin nuclear accumulation thereby increasing Wnt/β-catenin signalling (Lee et al., 2010, Kaler et al., 2009b, Brown et al., 2010). Similarly, the Western blot results showed upregulation of apical molecules of the PI3K/Akt and the Wnt/β-catenin. The results suggest that colitis associated activation of PI3K/Akt signalling activates the Wnt/β-catenin pathway by increasing p-GSK3β and directing β-catenin to the nucleus to upregulate transcription factors that induce cell proliferation and cell differentiation signalling.

A recent study demonstrated that colon cancer cells stimulate normal human monocytes and THP1 macrophages to release IL-1β, and showed that IL-1β is sufficient to induce canonical Wnt signalling and to promote growth of colon cancer cells through inactivation of GSK3β in the epithelial cells, establishing a previously unknown link among

inflammation, IL-1β and Wnt signalling and growth of colon cancer cells (Kaler et al., 2009a). Another study from the same group has shown that IL-1β secreted by tumour associated macrophages activate NF-κB dependent PDK1/AKT signalling which mediate phosphorylation of GSK3β and enhance activation of Wnt signalling, and promote c-myc. (Kaler et al., 2009b). Activation of NF-κB family of transcription factors in tumour cells appears to be a critical molecular link between inflammation and cancer (Karin and Greten, 2005). Activation of NF-*κ*B can stimulate tumour progression and invasion through the expression of VEGF, Cox-2, and IL-8 to promote angiogenesis (Wang et al., 2009).

Specific inactivation of NF-κB signalling in intestinal cells dramatically decreased the incidence of intestinal tumours in a mouse model of colitis associated cancer (Greten et al., 2004). Analysis of Winnie x *Nlrp3*$^{-/-}$ at the gene and protein level shows an over expression of IL-1β in the tumour associated colonic tissue. The above observations establish increased production of IL-1β and subsequent over activation of NF-κB leading to the aberrant activation of Wnt signalling as a novel molecular link between inflammation and tumor growth which is very relevant mechanism to the tumorigenesis of Winnie x *Nlrp3*$^{-/-}$.

Pten (Phosphatase and tensin), acts as a tumor suppressor gene, has been found damaged or deficient in many cancer (Lin et al., 2011, Garcia et al., 1999). One major function of PTEN is to negatively regulate PI3K signalling (Stambolic et al., 1998). The abnormal activation of Wnt/β-catenin signalling is one of the major causes of cancer, which can be regulated by PTEN/PI3K/Akt signalling through the phosphorylation of GSK-3β. The decreased expression of *Pten* gene could be responsible for the cell proliferation effect in Winnie x *Nlrp3*$^{-/-}$ colon cancer cells

The results suggest the deficiency of NLRP3 inflammasome stimulates NF-κB, PTEN/PI3K/Akt and Wnt/β-catenin signalling mechanisms which play central role in CAC seen in Winnie x *Nlrp3*$^{-/-}$ mouse model. However, the exact mechanism by which the NLRP3 inflammasome negatively regulates these pathways remains to be defined. Therefore, this model could be used to further study major pathways initiating colon cancer and could reveal potential therapeutic target for treatment for CAC.

Analysis of the gut microbiota composition shows Winnie x *Nlrp3*$^{-/-}$ mice promotes the development of a proinflammatory microbiota. The study found that the genus Prevotella, which have been shown to be colitogenic (Elinav et al., 2011) is significantly increased

whereas Lactobacillus a low risk associated with CAC (Reddy et al., 1985) is significantly decreased in the Winnie x *Nlrp3*$^{-/-}$. At the species level *Akkermansia muciniphila* is significantly increased in Winnie and Winnie x *Nlrp3*$^{-/-}$ mice microbiota. A recent study has shown the abundance of mucin-degrading microbe, pathobiont *Akkermansia muciniphila* was sufficient for promoting intestinal inflammation in spontaneous colitis in *Il10*$^{-/-}$ mice (Seregin et al., 2017). From these findings, it can be concluded that the overall nature of Winnie x *Nlrp3*$^{-/-}$ mice microbiota is intrinsically more pathogenic.

A healthy intestinal barrier is very important for intestinal homeostasis. This is underscored by the fact that any interference with the quality of mucus layer or with tight junctions leads to increased susceptibility to intestinal inflammation (Johansson et al., 2008, Petersson et al., 2010). Similarly, in Winnie mice, a missense mutation in the *Muc2* gene results in aberrant Muc2 biosynthesis leading to depleted mucus barrier increasing vulnerability to luminal bacteria, antigens and to inflammation-inducing toxins.

Furthermore, CAC is also associated with impaired barrier function, facilitating bacterial translocation and the induction of cytokines that maintain an inflammatory environment within the tumour (Jobin, 2012). Another study has detailed barrier deterioration induced during colon cancer results in adenoma invasion by microbial products, thereby promoting tumour-elicited inflammatory cytokines, including IL-17 and IL-23. These cytokines, in turn, lead to enhanced tumour growth (Grivennikov et al., 2012).

Consistent with these studies ablation of *Muc2,* a major component of mucus layer in the colon, in the *Muc2*$^{-/-}$ mice, causes spontaneous intestinal inflammation (Van der Sluis et al., 2006) that progresses to CAC even without introduction of additional mutations or carcinogens (Velcich et al., 2002) and also accelerates adenoma growth when introduced into *Apc*^{Min} mice (Yang et al., 2008).

From the studies mentioned above, we can conclude that a possible mechanism of pathogenesis of CAC observed in Winnie x *Nlrp3*$^{-/-}$ could be due to a depleted mucosal barrier exposing the intestinal epithelium to the tumorigenic bacteria identified in the Winnie x *Nlrp3*$^{-/-}$ microbiota in the absence of the protective innate immune modulator the NLRP3 Inflammasome.

The gut microbiota produces SCFA, which promote colonic health and ameliorate inflammation (Smith et al., 2013). The functions of butyrate in promoting colonic health

range from being energy source for colonocytes and by playing a role in the prevention and treatment of distal ulcerative colitis, Crohn's disease, and cancer (Canani et al., 2011). Gut microbiome analysis has revealed a significant decrease in the number of butyrate-producing bacteria in colon of patients with ulcerative colitis and colon cancer (Frank et al., 2007, Wang et al., 2012). Furthermore, colonic irrigation with butyrate suppresses inflammation during ulcerative colitis (Hamer et al., 2008). From the studies mentioned above we can conclude that the significant decrease in SCFAs particularly butyrate seen in the disease genotypes Winnie and Winnie x *Nlrp3*$^{-/-}$ contributes to the ulcerative colitis and colitis associated cancer phenotypes, respectively.

6.5 Conclusion

The data generated in this study showed that in Winnie x *Nlrp3*$^{-/-}$, molecules related to cell proliferation, angiogenesis, and anti-apoptotic activity were upregulated at the gene and protein level while down regulated molecules were negatively associated with colitis associated colon cancer. Altogether the results validate Winnie x *Nlrp3*$^{-/-}$ as a CAC model.

These dysregulated biomarkers points to a potential mechanism where NF-κB, PTEN/PI3K/Akt and Wnt/β-catenin signalling pathways play a central role in CAC seen in Winnie x *Nlrp3*$^{-/-}$ mouse model (Figure 6.10). Winnie x *Nlrp3*$^{-/-}$ has shown an association between colitogenic microbiota and CAC susceptibility and introduces a novel therapeutic marker for diagnosis or treatment of CAC in future. Furthermore, the Winnie x *Nlrp3*$^{-/-}$ mouse model introduced in the study represent a promising animal model for colitis associated colorectal cancer, caused by dysregulation in innate immune signalling, providing a new option for future studies in therapeutic agents for CAC.

In summary, the results of this study and those by others demonstrated that NLRP3 contributes to the negative regulation of tumorigenesis in CAC (Zaki et al., 2010b, Allen et al., 2010, Yao et al., 2016). Future studies unravelling the exact functional mechanism of NLRP3 in tumorigenesis could open new avenues in the treatment of colitis associated colon cancer.

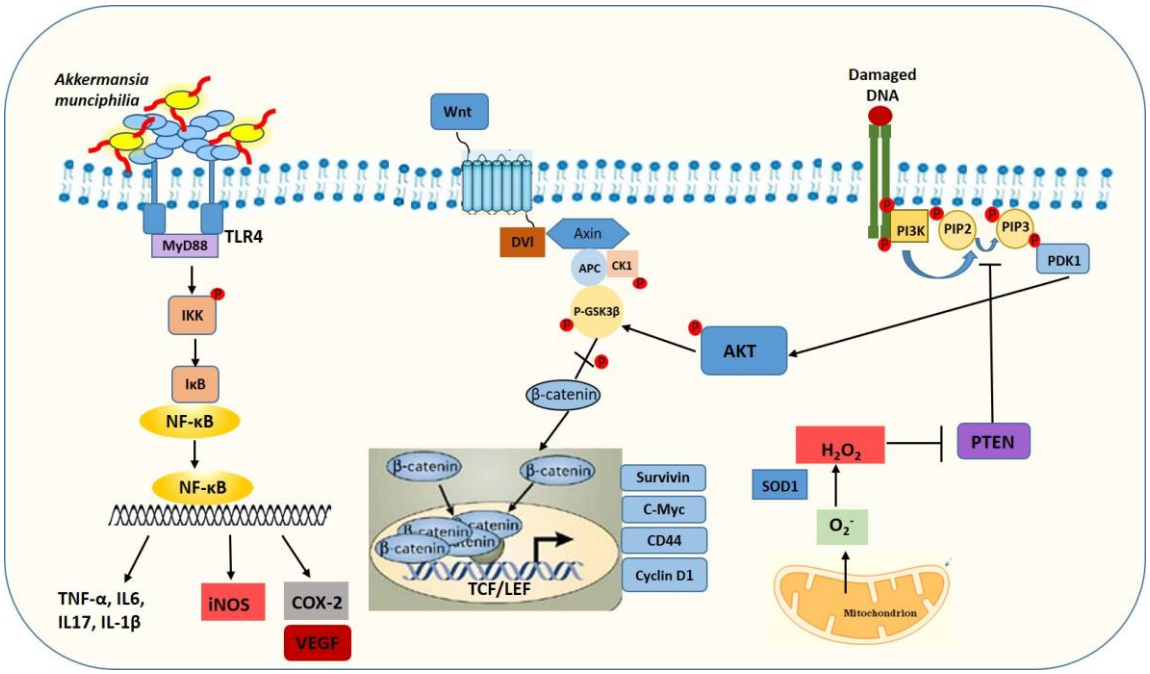


Figure 6.10: Proposed mechanism of colitis associated colon cancer in Winnie x *Nlrp3*$^{-/-}$ mouse model. The three signaling pathways controlling the tumorigenesis of the intestinal epithelium are Wnt, PI3K and NF-kB. 3-phosphinostitide dependent protein kinase-1 (PDK1), Casein Kinase 1 (CK1), Dishevelled protein (DVI), Glycogen synthase kinase- 3β (GSK3β),IκB kinase (IKK), Nuclear facto kB (NF-KB), Phosphate and tensin homolog deleted on chromosome 10 (PTEN), phosphatidylinositol 3-kinase (PI3K), T cell factor (TCF).

Chapter 7

General Discussion

7.1 Final Discussion

The inflammasome was first discovered by Martinon *et al.* (Martinon et al., 2002) and during the prevailing 17 years has been an intense area of research in immunology. NLRP3 inflammasome has been the most characterised among the inflammasomes and is closely associated with intestinal inflammatory diseases such as colitis. A major complication of colitis is the development of colitis-associated colorectal cancer (CAC). However, specific underlying mechanisms through which colitis regulates CAC initiation and progression are not understood.

Three main studies were conducted in this thesis; 1) investigate the efficacy of specific NLRP3 inhibitor MCC950 to ameliorate colitis (Chapter 4), 2) Evaluate the effect of NLRP3 on the severity of colitis, by the generation and study of Winnie mice deficient in *Nlrp3* (Chapter 5), 3) investigate the influence of NLRP3 inflammasome on gut microbiome and metabolomics in colitis (Chapter 6).

The reasons for conducting these studies were, that despite over 10 years of intensive research, the suggested role of NLRP3 in the gut remains multifaceted and controversial. Some studies using artificially induced models of colitis (Dextran Sodium Sulphate) and colorectal cancer (carcinogen Azoxymethane) indicate that *Nlrp3*$^{-/-}$ mice exhibit severe gut inflammation while others have reported protection from colitis and improved gut barrier integrity. The reason for the discrepancies observed in the *Nlrp3*$^{-/-}$ chemically induced colitis and CAC model phenotype is not clear but could be due to differences in length and concentration of chemical treatment or baseline differences in the composition of the intestinal microbiota in experimental mice.

To settle these conflicting data and as a novel solution to previous experimental errors, I conducted all investigations in the spontaneous colitis mouse model Winnie. Winnie mice have a single missense mutation in the *Muc2* mucin gene resulting in a depleted intestinal mucus barrier and develop spontaneous distal intestinal inflammation displaying symptoms of pain, diarrhoea and rectal bleeding at different stages of colitis like human ulcerative colitis (UC).

Currently, treatment for colitis is not curative; with patients commonly, enduring relapsing illness for the remainder of their lives. Thus, there is an urgent need to develop potent drugs with novel mechanisms of action. Given the evidence that aberrant NLRP3 activation is involved in the progression of colitis, targeting the activation pathway is a promising strategy for the development of novel effective therapeutics for colitis. Many chemical NLPR3 inhibitors tested in experimental colitis mouse models have shown reduced disease severity however these inhibitors were not specific to the NLRP3 inflammasome. MCC950 is a potent, highly specific small molecule inhibitor of both canonical and noncanonical activation of NLRP3 inflammasome and has been evaluated in a multitude of NLRP3 driven inflammatory diseases.

I investigated the therapeutic effect of MCC950 in the spontaneous chronic colitis mouse model Winnie. This study is significant, and, to my knowledge, it is the first to examine the efficacy of a specific NLRP3 inhibitor in a naturally occurring spontaneous colitis murine model. Application of a specific inhibitor enabled me to quantify the contribution of anti-inflammatory effects resulting exclusively from inhibition of both the canonical and non-canonical NLRP3 inflammasome activation in colitis. This primary finding supports the use of specific NLRP3 inhibitors as therapeutic option in colitis.

MCC950 treatment significantly ameliorated colitis with improved body weight gain, colon length, ratio of colon weight to body weight, and disease activity index. Histopathological scores of MCC950 treated Winnie mice were significantly reduced suggesting not only attenuation of ongoing colitis but also delay of disease onset. MCC950 significantly suppressed IL-1β and IL-18 cytokine expression at both mRNA and protein levels in Winnie colons. Additionally, MCC950 also effectively suppressed the release of proinflammatory cytokines and chemokines in mucosal explants. Furthermore, MCC950 treatment resulted in a significant decrease of IL-1β release and activation of caspase-1 in Winnie explants and *in vitro* macrophage cells isolated from Winnie mice. Collectively, our results illustrate the efficacy of MCC950 in the treatment of murine ulcerative colitis and has clinically relevant implications as a novel therapeutic agent for human inflammatory intestinal diseases.

Moreover, I investigated the possibility of MCC950 and Glyburide target to be the NEK7-NLRP3 interaction. Shi et al has shown that NEK7 phosphorylation enhances its binding to

NLRP3 and promotes inflammasome activation (Shi et al., 2016). Our results showed no difference in the level of phosphorylated NEK7 between the untreated and MCC950 or glyburide treated samples. This result suggest that the inhibitory target is not NEK7-NLRP3 but is downstream of this interaction. This finding will significantly add to the current understanding of the inhibitory mechanism of MCC950.

With the success in chemically inhibiting NLRP3 and ameliorating colitis I hypothesized that genetic ablation of *Nlrp3* gene will cure the colonic inflammation in Winnie mice. Thus, to take the research a step further, a novel mouse model was generated by knocking out the *Nlrp3* gene in the spontaneous colitis mouse model Winnie, within a defined microbiota. Extensive studies done in Winnie has proven it to be a suitable murine model to study colitis and its pathogenesis. The inflammatory pathology of Winnie mice has been analysed up to one year of age and a spontaneous development of CAC has not been observed. To my knowledge, this study is the first to examine the role of NLRP3 in inflammation, associated with a naturally occurring spontaneous colitis without the need for carcinogen treatment, similar to CAC pathogenesis in humans.

The detailed phenotypical analysis of Winnie x *Nlrp3*$^{-/-}$ colon at 12 and 16 weeks showed spontaneous multiple colonic tumours. Winnie x *Nlrp3*$^{-/-}$ mice had significantly shorter colons, and a higher ratio of colon weight to length and poorer survival rate than the control groups indicating the severity of colitis and tumorigenesis. Histopathology of Winnie x *Nlrp3*$^{-/-}$ colon shows biological features like colitis associated colorectal dysplasia–invasive carcinoma sequence observed in human CAC and in future will be a useful experimental model for anti CAC therapeutic agents.

The study analysed the expression of bio-markers *Bcl2, Sod2, Pparγ, Myc, Birc5* and *Cdk2* and their upregulation was positively related to colonic tumorigenesis. These findings could be potentially useful in identifying early prognostic bio-markers in CAC.

Winnie x *Nlrp3*$^{-/-}$ mice colonic tissue and serum analysis showed an elevation of proinflammatory cytokines, MPO, iNOS, CRP, ROS and DNA damage biomarkers. These biomarkers are positively associated with decreased survival of CAC patients and explains the poor survival rate of Winnie x *Nlrp3*$^{-/-}$ mice.

Immunofluorescence data generated in this study showed that in Winnie x *Nlrp3*$^{-/-}$, molecules related to cell proliferation, angiogenesis, and anti-apoptotic activity were

upregulated at the gene and protein level while down regulated molecules were negatively associated with colitis associated colon cancer. The molecular mechanism of tumorigenesis was revealed by protein analysis of colonic tumours and Western blot results showed upregulation of PI3K/ AKT and Wnt/β-catenin pathways. Collectively, these results validate Winnie x *Nlrp3*$^{-/-}$ as a suitable CAC murine model.

The thesis reports novel findings from the analysis of the microbiota and metabolites in Winnie x *Nlrp3*$^{-/-}$ mice colon to define the role of NLRP3 inflammasome in CAC penetrance. Faecal microbiota analysis revealed significant increase in colitogenic members in the phylogenetic architecture in Winnie x *Nlrp3*$^{-/-}$ mice while metabolomics profiling revealed upregulation of key metabolites and short chain fatty acids. These results confirmed the role of NLRP3 as a negative regulator of tumorigenesis during CAC.

The primary result of this study has provided the first evaluation of a specific NLRP3 inhibitor in colitis and will allow potentially new therapeutic approaches to emerge. Altogether, results of this thesis will significantly add to the current understanding of the importance of NLRP3 in pathogenesis and pathophysiology of colitis and CAC.

The main limitation of these studies was that all experiments were performed in mouse models and cannot be directly translated to clinical utility. Fortunately, there is a striking concordance in the human and mouse inflammasome complexes. However, recent research showed that there are changes in types of receptors, caspases and regulatory mechanisms which clearly warrants further studies in patient samples.

7.2 Future directions

The above discussion highlights the crucial importance of using human macrophage and colonic tissue samples from individuals with active ulcerative colitis to validate the therapeutic effect of MCC950 observed in the experimental mouse model. A positive outcome will benefit to optimize the current therapeutic regime in IBD.

The earliest time point the Winnie x *Nlrp3*$^{-/-}$ colon was examined was at week 12 and the results highlight key mechanisms in advanced stage of CAC rather than the early stages of inflammation driven tumorigenesis. Therefore, there is a need for in depth studies at an early age to delineate the critical underlying mucosal and immune cell mediated mechanisms involved in the tipping point from IBD to CAC. To this end, a future study can employ advanced cell culture techniques such as three-dimensional (3D) organoid

cultures derived from stem cell-containing colon or tumoroid. These organoid cultures are far superior to 2D cell culture and can be used to evaluate specific mechanisms ex vivo as well as inflammatory pathways on cell survival and proliferation. Additionally, patient-derived organoid cultures will allow for characterization and therapeutic evaluation for personalized care.

Another exciting innovation that can be incorporated to organoid culture is the genome editing novel technique CRISPR-Cas9. We propose to investigate the upregulated signalling pathways involved in the transformation of colitis to colorectal cancer using CRISPR-Cas9 genome editing in primary organoid cultures generated from CAC patient biopsies.

7.3 Concluding remarks

Colitis is a lifelong disease with no current cure. The investigations of this thesis show specific chemical inhibition of an over active NLRP3 inflammasome in chronic colitis attenuated severity of the disease. This indicates a harmful effect of an overactive NLRP3 inflammasome in colitis and the potential therapeutic intervention of NLRP3 inhibitors. The success MCC950 attenuating symptoms of IBD is an exciting therapeutic development with significant clinical translational value and should be acknowledged as such.

However, the genetic ablation of NLRP3 gene in a spontaneous chronic colitis model lead to a phenotype of colitis associated colorectal cancer. This highlights the critical function of NLRP3 inflammasome as a negative regulator of tumorigenesis in CAC. The novel model Winnie x *Nlrp3*$^{-/-}$ shows biological features like CAC dysplasia–invasive carcinoma sequence observed in human CAC and represent a promising mouse model for studying the molecular mechanism of CAC. Moreover, the Winnie x *Nlrp3*$^{-/-}$ model will also provide an experimental model to investigate the efficacy of therapeutic interventions by novel agents at different stages of colon cancer. The study introduces possible microbial and molecular interventions for CAC, giving this study a significant clinical relevance.

All together the results have generated new data and knowledge that defines the NLRP3 inflammasome as a double-edged sword in colitis and CAC. The results of this thesis highlight the complex roles of NLRP3 and stresses the importance of evaluating the dosage and long-term effect of novel NLRP3 inhibitors designed for chronic colitis in clinically

relevant experimental models before progressing to human clinical trials. In conclusion, as far as the gut is concerned NLRP3 activation should not be too hot or too cold but just rite!

References

ABBAS, T. & DUTTA, A. 2009. p21 in cancer: intricate networks and multiple activities. *Nat Rev Cancer,* 9**,** 400-14.

ACOSTA-RODRIGUEZ, E. V., NAPOLITANI, G., LANZAVECCHIA, A. & SALLUSTO, F. 2007. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol,* 8**,** 942-9.

ADAMKOV, M., VYBOHOVA, D., TUPA, V., CHYLIKOVA, J., HORACEK, J. & BENCAT, M. 2015. Expression and significance of survivin in colorectal high grade and low grade adenomas. *Acta Histochem,* 117**,** 590-4.

AKIRA, S., MISAWA, T., SATOH, T. & SAITOH, T. 2013. Macrophages control innate inflammation. *Diabetes Obes Metab,* 15 Suppl 3**,** 10-8.

ALLEN, I. C., SCULL, M. A., MOORE, C. B., HOLL, E. K., MCELVANIA-TEKIPPE, E., TAXMAN, D. J., GUTHRIE, E. H., PICKLES, R. J. & TING, J. P. 2009. The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA. *Immunity,* 30**,** 556-65.

ALLEN, I. C., TEKIPPE, E. M., WOODFORD, R. M., URONIS, J. M., HOLL, E. K., ROGERS, A. B., HERFARTH, H. H., JOBIN, C. & TING, J. P. 2010. The NLRP3 inflammasome functions as a negative regulator of tumorigenesis during colitis-associated cancer. *J Exp Med,* 207**,** 1045-56.

ALTIERI, D. C. 2003. Validating survivin as a cancer therapeutic target. *Nat Rev Cancer,* 3**,** 46-54.

AMARAL, F. A., COSTA, V. V., TAVARES, L. D., SACHS, D., COELHO, F. M., FAGUNDES, C. T., SORIANI, F. M., SILVEIRA, T. N., CUNHA, L. D., ZAMBONI, D. S., QUESNIAUX, V., PERES, R. S., CUNHA, T. M., CUNHA, F. Q., RYFFEL, B., SOUZA, D. G. & TEIXEIRA, M. M. 2012. NLRP3 inflammasome-mediated neutrophil recruitment and hypernociception depend on leukotriene B(4) in a murine model of gout. *Arthritis Rheum,* 64**,** 474-84.

ANAND, P. K., MALIREDDI, R. K., LUKENS, J. R., VOGEL, P., BERTIN, J., LAMKANFI, M. & KANNEGANTI, T. D. 2012. NLRP6 negatively regulates innate immunity and host defence against bacterial pathogens. *Nature,* 488**,** 389-93.

ANDERSON, M. G., NAKANE, M., RUAN, X., KROEGER, P. E. & WU-WONG, J. R. 2006. Expression of VDR and CYP24A1 mRNA in human tumors. *Cancer Chemother Pharmacol,* 57**,** 234-40.

ARAI, Y., TAKANASHI, H., KITAGAWA, H. & OKAYASU, I. 1998. Involvement of Interleukin-1 in the development of ulcerative colitis induced by DextranSulfate Sodium in mice. *Cytokine,* 10**,** 890-896.

ARPAIA, N., CAMPBELL, C., FAN, X., DIKIY, S., VAN DER VEEKEN, J., DEROOS, P., LIU, H., CROSS, J. R., PFEFFER, K., COFFER, P. J. & RUDENSKY, A. Y. 2013. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature,* 504**,** 451-5.

ARTHUR, J. C., PEREZ-CHANONA, E., MUHLBAUER, M., TOMKOVICH, S., URONIS, J. M., FAN, T. J., CAMPBELL, B. J., ABUJAMEL, T., DOGAN, B., ROGERS, A. B., RHODES, J. M., STINTZI, A., SIMPSON, K. W., HANSEN, J. J., KEKU, T. O., FODOR, A. A. & JOBIN, C. 2012. Intestinal inflammation targets cancer-inducing activity of the microbiota. *Science,* 338**,** 120-3.

ASQUITH, M. & POWRIE, F. 2010. An innately dangerous balancing act: intestinal homeostasis, inflammation, and colitis-associated cancer. *J Exp Med,* 207**,** 1573-7.

ATREYA, R., MUDTER, J., FINOTTO, S., MULLBERG, J., JOSTOCK, T., WIRTZ, S., SCHUTZ, M., BARTSCH, B., HOLTMANN, M., BECKER, C., STRAND, D., CZAJA, J., SCHLAAK, J. F., LEHR, H. A., AUTSCHBACH, F., SCHURMANN, G., NISHIMOTO, N., YOSHIZAKI, K., ITO, H., KISHIMOTO, T., GALLE, P. R., ROSE-JOHN, S. & NEURATH, M. F. 2000. Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis in vivo. *Nat Med,* 6**,** 583-8.

ATREYA, R. & NEURATH, M. F. 2005. Involvement of IL-6 in the pathogenesis of inflammatory bowel disease and colon cancer. *Clin Rev Allergy Immunol,* 28**,** 187-96.

ATREYA, R. & NEURATH, M. F. 2008. New therapeutic strategies for treatment of inflammatory bowel disease. *Mucosal Immunol,* 1**,** 175-82.

AUSTRALIA, C. A. C. A. O. 2013. CCA annual report 2012/2013. *www.crohnsandcolitis.com.au,* 1**,** 5.

BAKER, A. R., MCDONNELL, D. P., HUGHES, M., CRISP, T. M., MANGELSDORF, D. J., HAUSSLER, M. R., PIKE, J. W., SHINE, J. & O'MALLEY, B. W. 1988. Cloning and expression of full-length cDNA encoding human vitamin D receptor. *Proc Natl Acad Sci U S A,* 85**,** 3294-8.

BAKKER, W. J., HARRIS, I. S. & MAK, T. W. 2007. FOXO3a is activated in response to hypoxic stress and inhibits HIF1-induced apoptosis via regulation of CITED2. *Mol Cell,* 28**,** 941-53.

BARBER, M. D., POWELL, J. J., LYNCH, S. F., FEARON, K. C. & ROSS, J. A. 2000. A polymorphism of the interleukin-1 beta gene influences survival in pancreatic cancer. *Br J Cancer,* 83**,** 1443-7.

BAUER, C., DUEWELL, P., LEHR, H. A., ENDRES, S. & SCHNURR, M. 2012. Protective and aggravating effects of Nlrp3 inflammasome activation in IBD models: influence of genetic and environmental factors. *Dig Dis,* 30 Suppl 1**,** 82-90.

BAUER, C., DUEWELL, P., MAYER, C., LEHR, H. A., FITZGERALD, K. A., DAUER, M., TSCHOPP, J., ENDRES, S., LATZ, E. & SCHNURR, M. 2010. Colitis induced in mice with dextran sulfate sodium (DSS) is mediated by the NLRP3 inflammasome. *Gut,* 59**,** 1192-9.

BAUER, C., LOHER, F., DAUER, M., MAYER, C., LEHR, H. A., SCHÖNHARTING, M., HALLWACHS, R., ENDRES, S. & EIGLER, A. 2007. The ICE Inhibitor Pralnacasan Prevents DSS-Induced Colitis in C57BL/6 Mice and Suppresses IP-10 mRNA but Not TNF-α mRNA Expression. *Digestive Diseases and Sciences,* 52**,** 1642-1652.

BEALE, D. J., MARNEY, D., MARLOW, D. R., MORRISON, P. D., DUNN, M. S., KEY, C. & PALOMBO, E. A. 2013. Metabolomic analysis of Cryptosporidium parvum oocysts in water: a proof of concept demonstration. *Environ Pollut,* 174**,** 201-3.

BEALE, D. J., MORRISON, P. D., KEY, C. & PALOMBO, E. A. 2014. Metabolic profiling of biofilm bacteria known to cause microbial influenced corrosion. *Water Sci Technol,* 69**,** 1-8.

BEAUGERIE, L., BROUSSE, N., BOUVIER, A. M., COLOMBEL, J. F., LEMANN, M., COSNES, J., HEBUTERNE, X., CORTOT, A., BOUHNIK, Y., GENDRE, J. P., SIMON, T., MAYNADIE, M., HERMINE, O., FAIVRE, J. & CARRAT, F. 2009. Lymphoproliferative disorders in patients receiving thiopurines for inflammatory bowel disease: a prospective observational cohort study. *Lancet,* 374**,** 1617-25.

BECKER, C., FANTINI, M. C., SCHRAMM, C., LEHR, H. A., WIRTZ, S., NIKOLAEV, A., BURG, J., STRAND, S., KIESSLICH, R., HUBER, S., ITO, H., NISHIMOTO, N., YOSHIZAKI, K., KISHIMOTO, T., GALLE,

P. R., BLESSING, M., ROSE-JOHN, S. & NEURATH, M. F. 2004. TGF-beta suppresses tumor progression in colon cancer by inhibition of IL-6 trans-signaling. *Immunity,* 21**,** 491-501.

BECKER, C., FANTINI, M. C., WIRTZ, S., NIKOLAEV, A., LEHR, H. A., GALLE, P. R., ROSE-JOHN, S. & NEURATH, M. F. 2005. IL-6 signaling promotes tumor growth in colorectal cancer. *Cell Cycle,* 4**,** 217-20.

BELHAM, C., ROIG, J., CALDWELL, J. A., AOYAMA, Y., KEMP, B. E., COMB, M. & AVRUCH, J. 2003. A mitotic cascade of NIMA family kinases. Nercc1/Nek9 activates the Nek6 and Nek7 kinases. *J Biol Chem,* 278**,** 34897-909.

BENDARDAF, R., BUHMEIDA, A., HILSKA, M., LAATO, M., SYRJANEN, S., SYRJANEN, K., COLLAN, Y. & PYRHONEN, S. 2008. VEGF-1 expression in colorectal cancer is associated with disease localization, stage, and long-term disease-specific survival. *Anticancer Res,* 28**,** 3865-70.

BERG, D. J., DAVIDSON, N., KUHN, R., MULLER, W., MENON, S., HOLLAND, G., THOMPSON-SNIPES, L., LEACH, M. W. & RENNICK, D. 1996. Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. *J Clin Invest,* 98**,** 1010-20.

BERNSTEIN, C. N., WAJDA, A., SVENSON, L. W., MACKENZIE, A., KOEHOORN, M., JACKSON, M., FEDORAK, R., ISRAEL, D. & BLANCHARD, J. F. 2006. The epidemiology of inflammatory bowel disease in Canada: A population based study (vol 101, pg 1559, 2006). *American Journal of Gastroenterology,* 101**,** 1945-1945.

BERSUDSKY, M., LUSKI, L., FISHMAN, D., WHITE, R. M., ZIV-SOKOLOVSKAYA, N., DOTAN, S., RIDER, P., KAPLANOV, I., AYCHEK, T., DINARELLO, C. A., APTE, R. N. & VORONOV, E. 2014. Non-redundant properties of IL-1alpha and IL-1beta during acute colon inflammation in mice. *Gut,* 63**,** 598-609.

BETTELLI, E., CARRIER, Y., GAO, W., KORN, T., STROM, T. B., OUKKA, M., WEINER, H. L. & KUCHROO, V. K. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature,* 441**,** 235-8.

BIOQUE, G., CRUSIUS, J. B., KOUTROUBAKIS, I., BOUMA, G., KOSTENSE, P. J., MEUWISSEN, S. G. & PENA, A. S. 1995. Allelic polymorphism in IL-1 beta and IL-1 receptor antagonist (IL-1Ra) genes in inflammatory bowel disease. *Clin Exp Immunol,* 102**,** 379-83.

BLAZEJEWSKI, A. J., THIEMANN, S., SCHENK, A., PILS, M. C., GALVEZ, E. J. C., ROY, U., HEISE, U., DE ZOETE, M. R., FLAVELL, R. A. & STROWIG, T. 2017. Microbiota Normalization Reveals that Canonical Caspase-1 Activation Exacerbates Chemically Induced Intestinal Inflammation. *Cell Rep,* 19**,** 2319-2330.

BLUMBERG, R. & POWRIE, F. 2012. Microbiota, disease, and back to health: a metastable journey. *Sci Transl Med,* 4**,** 137rv7.

BLUMBERG, R. S. & STROBER, W. 2001. Prospects for research in inflammatory bowel disease. *Journal of the American Medical Association,* 285**,** 643–647.

BOLLRATH, J., PHESSE, T. J., VON BURSTIN, V. A., PUTOCZKI, T., BENNECKE, M., BATEMAN, T., NEBELSIEK, T., LUNDGREN-MAY, T., CANLI, O., SCHWITALLA, S., MATTHEWS, V., SCHMID, R. M., KIRCHNER, T., ARKAN, M. C., ERNST, M. & GRETEN, F. R. 2009. gp130-mediated Stat3 activation in enterocytes regulates cell survival and cell-cycle progression during colitis-associated tumorigenesis. *Cancer Cell,* 15**,** 91-102.

BOUMA, G. & STROBER, W. 2003. The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol,* 3**,** 521-533.

BRIERLEY, S. M. & LINDEN, D. R. 2014. Neuroplasticity and dysfunction after gastrointestinal inflammation. *Nat Rev Gastroenterol Hepatol,* 11**,** 611-27.

BROWN, J. B., LEE, G., MANAGLIA, E., GRIMM, G. R., DIRISINA, R., GORETSKY, T., CHERESH, P., BLATNER, N. R., KHAZAIE, K., YANG, G. Y., LI, L. & BARRETT, T. A. 2010. Mesalamine inhibits epithelial beta-catenin activation in chronic ulcerative colitis. *Gastroenterology,* 138**,** 595-605, 605.e1-3.

BURISCH, J., JESS, T., MARTINATO, M., LAKATOS, P. L. & EPICOM, E. 2013. The burden of inflammatory bowel disease in Europe. *Journal of Crohns & Colitis,* 7**,** 322-337.

CAI, Y., MA, W., HUANG, X., CAO, L., LI, H., JIANG, Y., LU, N. & YIN, Y. 2015. Effect of survivin on tumor growth of colorectal cancer in vivo. *International journal of clinical and experimental pathology,* 8**,** 13267-13272.

CAMELL, C., GOLDBERG, E. & DIXIT, V. D. 2015. Regulation of Nlrp3 inflammasome by dietary metabolites. *Semin Immunol,* 27**,** 334-42.

CANANI, R. B., COSTANZO, M. D., LEONE, L., PEDATA, M., MELI, R. & CALIGNANO, A. 2011. Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. *World journal of gastroenterology,* 17**,** 1519-1528.

CANAVAN, C., ABRAMS, K. R. & MAYBERRY, J. 2006. Meta-analysis: colorectal and small bowel cancer risk in patients with Crohn's disease. *Aliment Pharmacol Ther,* 23**,** 1097-104.

CAO, D., HOU, M., GUAN, Y. S., JIANG, M., YANG, Y. & GOU, H. F. 2009. Expression of HIF-1alpha and VEGF in colorectal cancer: association with clinical outcomes and prognostic implications. *BMC Cancer,* 9**,** 432.

CASINI-RAGGI, V., KAM, L., CHONG, Y. J., FIOCCHI, C., PIZARRO, T. T. & COMINELLI, F. 1995. Mucosal imbalance of IL-1 and IL-1 receptor antagonist in inflammatory bowel disease. A novel mechanism of chronic intestinal inflammation. *J Immunol,* 154**,** 2434-40.

CASTELL, J. V., GOMEZ-LECHON, M. J., DAVID, M., FABRA, R., TRULLENQUE, R. & HEINRICH, P. C. 1990. Acute-phase response of human hepatocytes: regulation of acute-phase protein synthesis by interleukin-6. *Hepatology,* 12**,** 1179-86.

CASTELLONE, M. D., TERAMOTO, H., WILLIAMS, B. O., DRUEY, K. M. & GUTKIND, J. S. 2005. Prostaglandin E2 promotes colon cancer cell growth through a Gs-axin-beta-catenin signaling axis. *Science,* 310**,** 1504-10.

CHAE, W.-J., GIBSON, T. F., ZELTERMAN, D., HAO, L., HENEGARIU, O. & BOTHWELL, A. L. M. 2010. Ablation of IL-17A abrogates progression of spontaneous intestinal tumorigenesis. *Proceedings of the National Academy of Sciences of the United States of America,* 107**,** 5540-5544.

CHAIX, J., TESSMER, M. S., HOEBE, K., FUSERI, N., RYFFEL, B., DALOD, M., ALEXOPOULOU, L., BEUTLER, B., BROSSAY, L., VIVIER, E. & WALZER, T. 2008. Cutting edge: Priming of NK cells by IL-18. *J Immunol,* 181**,** 1627-31.

CHAO, C., ZHANG, Z. F., BERTHILLER, J., BOFFETTA, P. & HASHIBE, M. 2006. NAD(P)H:quinone oxidoreductase 1 (NQO1) Pro187Ser polymorphism and the risk of lung, bladder, and colorectal cancers: a meta-analysis. *Cancer Epidemiol Biomarkers Prev,* 15**,** 979-87.

CHASSAING, B., AITKEN, J. D., MALLESHAPPA, M. & VIJAY-KUMAR, M. 2014. Dextran sulfate sodium (DSS)-induced colitis in mice. *Curr Protoc Immunol,* 104**,** Unit 15.25.

CHAUDHRY, A., SAMSTEIN, R. M., TREUTING, P., LIANG, Y., PILS, M. C., HEINRICH, J. M., JACK, R. S., WUNDERLICH, F. T., BRUNING, J. C., MULLER, W. & RUDENSKY, A. Y. 2011. Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. *Immunity,* 34**,** 566-78.

CHAVARRIA-SMITH, J. & VANCE, R. E. 2015. The NLRP1 inflammasomes. *Immunol Rev,* 265**,** 22-34.

CHEN, G. Y. & NUNEZ, G. 2011. Inflammasomes in intestinal inflammation and cancer. *Gastroenterology,* 141**,** 1986-99.

CHEN, Y., HUI, H., YANG, H., ZHAO, K., QIN, Y., GU, C., WANG, X., LU, N. & GUO, Q. 2013. Wogonoside induces cell cycle arrest and differentiation by affecting expression and subcellular localization of PLSCR1 in AML cells. *Blood,* 121**,** 3682-91.

CHEN, Y., LU, N., LING, Y., GAO, Y., WANG, L., SUN, Y., QI, Q., FENG, F., LIU, W., LIU, W., YOU, Q. & GUO, Q. 2009. Wogonoside inhibits lipopolysaccharide-induced angiogenesis in vitro and in vivo via toll-like receptor 4 signal transduction. *Toxicology,* 259**,** 10-7.

CHOUHAN, S., GAHLOT, S., POKHARNA, R. K., MATHUR, K. C., SAINI, K. & PAL, M. 2006. Severity and extent of ulcerative colitis: role of C-reactive protein. *Indian J Gastroenterol,* 25**,** 46-7.

CHUNG, Y., CHANG, S. H., MARTINEZ, G. J., YANG, X. O., NURIEVA, R., KANG, H. S., MA, L., WATOWICH, S. S., JETTEN, A. M., TIAN, Q. & DONG, C. 2009. Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. *Immunity,* 30**,** 576-87.

CHUNG, Y. C. & CHANG, Y. F. 2003. Serum interleukin-6 levels reflect the disease status of colorectal cancer. *J Surg Oncol,* 83**,** 222-6.

CIMA, R. R. 2010. Timing and indications for colectomy in chronic ulcerative colitis: Surgical consideration. *Dig Dis,* 28**,** 501-7.

CLAMBEY, E. T., MCNAMEE, E. N., WESTRICH, J. A., GLOVER, L. E., CAMPBELL, E. L., JEDLICKA, P., DE ZOETEN, E. F., CAMBIER, J. C., STENMARK, K. R., COLGAN, S. P. & ELTZSCHIG, H. K. 2012. Hypoxia-inducible factor-1 alpha-dependent induction of FoxP3 drives regulatory T-cell abundance and function during inflammatory hypoxia of the mucosa. *Proc Natl Acad Sci U S A,* 109**,** E2784-93.

COCCIA, M., HARRISON, O. J., SCHIERING, C., ASQUITH, M. J., BECHER, B., POWRIE, F. & MALOY, K. J. 2012. IL-1beta mediates chronic intestinal inflammation by promoting the accumulation of IL-17A secreting innate lymphoid cells and CD4(+) Th17 cells. *J Exp Med,* 209**,** 1595-609.

COLL, R. C., ROBERTSON, A. A. B., CHAE, J. J., HIGGINS, S. C., MUNOZ-PLANILLO, R., INSERRA, M. C., VETTER, I., DUNGAN, L. S., MONKS, B. G., STUTZ, A., CROKER, D. E., BUTLER, M. S., HANEKLAUS, M., SUTTON, C. E., NUNEZ, G., LATZ, E., KASTNER, D. L., MILLS, K. H. G., MASTERS, S. L., SCHRODER, K., COOPER, M. A. & O'NEILL, L. A. J. 2015. A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases. *Nat Med,* 21**,** 248-255.

COLOTTA, F., ALLAVENA, P., SICA, A., GARLANDA, C. & MANTOVANI, A. 2009. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis,* 30**,** 1073-1081.

CONTU, P. D. C., CONTU, S. S., ROSITO, M. A. & MOREIRA, L. F. 2009. Expression of C-Reactive Protein in Rectal Cancer. *Gastroenterology research,* 2**,** 104-109.

COONEY, R. V., CHAI, W., FRANKE, A. A., WILKENS, L. R., KOLONEL, L. N. & LE MARCHAND, L. 2013. C-reactive protein, lipid-soluble micronutrients, and survival in colorectal cancer patients. *Cancer Epidemiol Biomarkers Prev,* 22**,** 1278-88.

COOPER, H. S., MURTHY, S., KIDO, K., YOSHITAKE, H. & FLANIGAN, A. 2000. Dysplasia and cancer in the dextran sulfate sodium mouse colitis model. Relevance to colitis-associated neoplasia in the human: a study of histopathology, B-catenin and p53 expression and the role of inflammation. *Carcinogenesis,* 21**,** 757-68.

COSNES, J., GOWER-ROUSSEAU, C., SEKSIK, P. & CORTOT, A. 2011. Epidemiology and natural history of inflammatory bowel diseases. *Gastroenterology,* 140**,** 1785-94.

COSTA, C., INCIO, J. & SOARES, R. 2007. Angiogenesis and chronic inflammation: cause or consequence? *Angiogenesis,* 10**,** 149-66.

CROHN, B. & ROSENBERG, H. 1925a. he sigmoidoscopic picture of chronic ulcerative colitis (non-specific). *American Journal of Medicle Sciences,* 170**,** 220–228.

CROHN, B. B. & ROSENBERG, H. 1925b. THE SIGMOIDOSCOPIC PICTURE OF CHRONIC ULCERATIVE COLITIS (NON-SPECIFIC). *The American Journal of the Medical Sciences,* 170.

CRUZ, C. M., RINNA, A., FORMAN, H. J., VENTURA, A. L., PERSECHINI, P. M. & OJCIUS, D. M. 2007. ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages. *J Biol Chem,* 282**,** 2871-9.

CURTIN, N. J. 2012. DNA repair dysregulation from cancer driver to therapeutic target. *Nature Reviews Cancer,* 12**,** 801.

D'INCA, R., CARDIN, R., BENAZZATO, L., ANGRIMAN, I., MARTINES, D. & STURNIOLO, G. C. 2004. Oxidative DNA damage in the mucosa of ulcerative colitis increases with disease duration and dysplasia. *Inflamm Bowel Dis,* 10**,** 23-7.

DANG, C. V. 2012. MYC on the path to cancer. *Cell,* 149**,** 22-35.

DANIELSEN, S. A., EIDE, P. W., NESBAKKEN, A., GUREN, T., LEITHE, E. & LOTHE, R. A. 2015. Portrait of the PI3K/AKT pathway in colorectal cancer. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer,* 1855**,** 104-121.

DANILEWICZ, M., STASIKOWSKA-KANICKA, O. & WAGROWSKA-DANILEWICZ, M. 2015. Augmented immunoexpression of survivin correlates with parameters of aggressiveness in prostate cancer. *Pol J Pathol,* 66**,** 44-8.

DAS, I., PNG, C. W., OANCEA, I., HASNAIN, S. Z., LOURIE, R., PROCTOR, M., ERI, R. D., SHENG, Y., CRANE, D. I., FLORIN, T. H. & MCGUCKIN, M. A. 2013. Glucocorticoids alleviate intestinal ER stress by enhancing protein folding and degradation of misfolded proteins. *Journal of Experimental Medicine,* 210**,** 1201-1216.

DASHDORJ, A., JYOTHI, K. R., LIM, S., JO, A., NGUYEN, M. N., HA, J., YOON, K. S., KIM, H. J., PARK, J. H., MURPHY, M. P. & KIM, S. S. 2013. Mitochondria-targeted antioxidant MitoQ ameliorates experimental mouse colitis by suppressing NLRP3 inflammasome-mediated inflammatory cytokines. *BMC Med,* 11**,** 178.

DAVIDSON, N. J., LEACH, M. W., FORT, M. M., THOMPSON-SNIPES, L., KUHN, R., MULLER, W., BERG, D. J. & RENNICK, D. M. 1996. T helper cell 1-type CD4+ T cells, but not B cells, mediate colitis in interleukin 10-deficient mice. *J Exp Med,* 184**,** 241-51.

DE SANTIS, S., KUNDE, D., GALLEGGIANTE, V., LISO, M., SCANDIFFIO, L., SERINO, G., PINTO, A., CAMPIGLIA, P., SORRENTINO, R., CAVALCANTI, E., SANTINO, A., CARUSO, M. L., ERI, R. &

CHIEPPA, M. 2017. TNFalpha deficiency results in increased IL-1beta in an early onset of spontaneous murine colitis. *Cell Death Dis,* 8**,** e2993.

DEN BESTEN, G., VAN EUNEN, K., GROEN, A. K., VENEMA, K., REIJNGOUD, D.-J. & BAKKER, B. M. 2013. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *Journal of lipid research,* 54**,** 2325-2340.

DENNIS, K. L., SAADALLA, A., BLATNER, N. R., WANG, S., VENKATESWARAN, V., GOUNARI, F., CHEROUTRE, H., WEAVER, C. T., ROERS, A., EGILMEZ, N. K. & KHAZAIE, K. 2015. T-cell Expression of IL10 Is Essential for Tumor Immune Surveillance in the Small Intestine. *Cancer Immunol Res,* 3**,** 806-14.

DESAI, M. S., SEEKATZ, A. M., KOROPATKIN, N. M., KAMADA, N., HICKEY, C. A., WOLTER, M., PUDLO, N. A., KITAMOTO, S., TERRAPON, N., MULLER, A., YOUNG, V. B., HENRISSAT, B., WILMES, P., STAPPENBECK, T. S., NUNEZ, G. & MARTENS, E. C. 2016. A Dietary Fiber-Deprived Gut Microbiota Degrades the Colonic Mucus Barrier and Enhances Pathogen Susceptibility. *Cell,* 167**,** 1339-1353.e21.

DEUIS, J. R., YIN, K., COOPER, M. A., SCHRODER, K. & VETTER, I. 2017. Role of the NLRP3 inflammasome in a model of acute burn-induced pain. *Burns,* 43**,** 304-309.

DHARIWAL, A., CHONG, J., HABIB, S., KING, I. L., AGELLON, L. B. & XIA, J. 2017. MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic acids research,* 45**,** W180-W188.

DINARELLO, C. A. 2009. Immunological and inflammatory functions of the interleukin-1 family. *Annu Rev Immunol,* 27**,** 519-50.

DINH, Q. N., DRUMMOND, G. R., KEMP-HARPER, B. K., DIEP, H., DE SILVA, T. M., KIM, H. A., VINH, A., ROBERTSON, A. A. B., COOPER, M. A., MANSELL, A., CHRISSOBOLIS, S. & SOBEY, C. G. 2017. Pressor response to angiotensin II is enhanced in aged mice and associated with inflammation, vasoconstriction and oxidative stress. *Aging-Us,* 9**,** 1595-1606.

DOSTERT, C., PETRILLI, V., VAN BRUGGEN, R., STEELE, C., MOSSMAN, B. T. & TSCHOPP, J. 2008. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science,* 320**,** 674-7.

DU, Q. M., WANG, Q., FAN, H. M., WANG, J. N., LIU, X. T., WANG, H., WANG, Y. J. & HU, R. 2016. Dietary cholesterol promotes AOM-induced colorectal cancer through activating the NLRP3 inflammasome. *Biochemical Pharmacology,* 105**,** 42-54.

DUEWELL, P., KONO, H., RAYNER, K. J., SIROIS, C. M., VLADIMER, G., BAUERNFEIND, F. G., ABELA, G. S., FRANCHI, L., NUNEZ, G., SCHNURR, M., ESPEVIK, T., LIEN, E., FITZGERALD, K. A., ROCK, K. L., MOORE, K. J., WRIGHT, S. D., HORNUNG, V. & LATZ, E. 2010. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature,* 464**,** 1357-61.

DUPAUL-CHICOINE, J., ARABZADEH, A., DAGENAIS, M., DOUGLAS, T., CHAMPAGNE, C., MORIZOT, A., RODRIGUE-GERVAIS, I. G., BRETON, V., COLPITTS, S. L., BEAUCHEMIN, N. & SALEH, M. 2015. The Nlrp3 Inflammasome Suppresses Colorectal Cancer Metastatic Growth in the Liver by Promoting Natural Killer Cell Tumoricidal Activity. *Immunity,* 43**,** 751-763.

DUPAUL-CHICOINE, J., YERETSSIAN, G., DOIRON, K., BERGSTROM, K. S., MCINTIRE, C. R., LEBLANC, P. M., MEUNIER, C., TURBIDE, C., GROS, P., BEAUCHEMIN, N., VALLANCE, B. A. & SALEH, M. 2010. Control of intestinal homeostasis, colitis, and colitis-associated colorectal cancer by the inflammatory caspases. *Immunity,* 32**,** 367-78.

EADEN, J. A., ABRAMS, K. R. & MAYBERRY, J. F. 2001. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut,* 48**,** 526-35.

EASWARAN, V., LEE, S. H., INGE, L., GUO, L., GOLDBECK, C., GARRETT, E., WIESMANN, M., GARCIA, P. D., FULLER, J. H., CHAN, V., RANDAZZO, F., GUNDEL, R., WARREN, R. S., ESCOBEDO, J., AUKERMAN, S. L., TAYLOR, R. N. & FANTL, W. J. 2003. beta-Catenin regulates vascular endothelial growth factor expression in colon cancer. *Cancer Res,* 63**,** 3145-53.

EDGAR, R. C. 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods,* 10**,** 996-8.

EDGAR, R. C., HAAS, B. J., CLEMENTE, J. C., QUINCE, C. & KNIGHT, R. 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics,* 27**,** 2194-2200.

EL-OMAR, E. M., CARRINGTON, M., CHOW, W. H., MCCOLL, K. E., BREAM, J. H., YOUNG, H. A., HERRERA, J., LISSOWSKA, J., YUAN, C. C., ROTHMAN, N., LANYON, G., MARTIN, M., FRAUMENI, J. F., JR. & RABKIN, C. S. 2001. The role of interleukin-1 polymorphisms in the pathogenesis of gastric cancer. *Nature,* 412**,** 99.

ELINAV, E., STROWIG, T., KAU, ANDREW L., HENAO-MEJIA, J., THAISS, CHRISTOPH A., BOOTH, CARMEN J., PEAPER, DAVID R., BERTIN, J., EISENBARTH, STEPHANIE C., GORDON, JEFFREY I. & FLAVELL, RICHARD A. 2011. NLRP6 Inflammasome Regulates Colonic Microbial Ecology and Risk for Colitis. *Cell,* 145**,** 745-757.

ELINAV, E., THAISS, C. A. & FLAVELL, R. A. 2013. Analysis of microbiota alterations in inflammasome-deficient mice. *Methods Mol Biol,* 1040**,** 185-94.

ENGEL, M. A. & NEURATH, M. F. 2010. Anticancer properties of the IL-12 family--focus on colorectal cancer. *Curr Med Chem,* 17**,** 3303-8.

ERI, R., MCGUCKIN, M. A. & WADLEY, R. 2012. T cell transfer model of colitis: a great tool to assess the contribution of T cells in chronic intestinal inflammation. *Methods Mol Biol,* 844**,** 261-75.

ERI, R. D., ADAMS, R. J., TRAN, T. V., TONG, H., DAS, I., ROCHE, D. K., OANCEA, I., PNG, C. W., JEFFERY, P. L., RADFORD-SMITH, G. L., COOK, M. C., FLORIN, T. H. & MCGUCKIN, M. A. 2011. An intestinal epithelial defect conferring ER stress results in inflammation involving both innate and adaptive immunity. *Mucosal Immunol,* 4**,** 354-64.

FANG, J., SEKI, T. & MAEDA, H. 2009. Therapeutic strategies by modulating oxygen stress in cancer and inflammation. *Adv Drug Deliv Rev,* 61**,** 290-302.

FAUBION, W. A., JR., LOFTUS, E. V., JR., HARMSEN, W. S., ZINSMEISTER, A. R. & SANDBORN, W. J. 2001. The natural history of corticosteroid therapy for inflammatory bowel disease: a population-based study. *Gastroenterology,* 121**,** 255-60.

FENG, J., PARK, J., CRON, P., HESS, D. & HEMMINGS, B. A. 2004. Identification of a PKB/Akt hydrophobic motif Ser-473 kinase as DNA-dependent protein kinase. *J Biol Chem,* 279**,** 41189-96.

FERLAY, J., SOERJOMATARAM, I., DIKSHIT, R., ESER, S., MATHERS, C., REBELO, M., PARKIN, D. M., FORMAN, D. & BRAY, F. 2015. Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. *International Journal of Cancer,* 136**,** E359-E386.

FERNANDES-ALNEMRI, T., YU, J. W., DATTA, P., WU, J. & ALNEMRI, E. S. 2009. AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. *Nature,* 458**,** 509-13.

FLEET, J. C., DESMET, M., JOHNSON, R. & LI, Y. 2012. Vitamin D and cancer: a review of molecular mechanisms. *Biochemical Journal,* 441**,** 61.

FOERSCH, S. & NEURATH, M. F. 2014. Colitis-associated neoplasia: molecular basis and clinical translation. *Cellular and Molecular Life Sciences,* 71**,** 3523-3535.

FORNARO, R., CARATTO, M., BARBRUNI, G., FORNARO, F., SALERNO, A., GIOVINAZZO, D., STICCHI, C. & CARATTO, E. 2015. Surgical and medical treatment in patients with acute severe ulcerative colitis. *J Dig Dis,* 16**,** 558-67.

FRANCHI, L., MUNOZ-PLANILLO, R. & NUNEZ, G. 2012. Sensing and reacting to microbes through the inflammasomes. *Nature Immunology,* 13**,** 325-332.

FRANK, D. N., ST AMAND, A. L., FELDMAN, R. A., BOEDEKER, E. C., HARPAZ, N. & PACE, N. R. 2007. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci U S A,* 104**,** 13780-5.

FRENCH, K. E., HARVEY, J. & MCCULLAGH, J. S. O. 2018. Targeted and Untargeted Metabolic Profiling of Wild Grassland Plants identifies Antibiotic and Anthelmintic Compounds Targeting Pathogen Physiology, Metabolism and Reproduction. *Scientific Reports,* 8**,** 1695.

FUJINO, S., ANDOH, A., BAMBA, S., OGAWA, A., HATA, K., ARAKI, Y., BAMBA, T. & FUJIYAMA, Y. 2003. Increased expression of interleukin 17 in inflammatory bowel disease. *Gut,* 52**,** 65.

FURUHASHI, T., SUGITATE, K., NAKAI, T., JIKUMARU, Y. & ISHIHARA, G. 2018. Rapid profiling method for mammalian feces short chain fatty acids by GC-MS. *Analytical biochemistry,* 543**,** 51-54.

FURUSAWA, Y., OBATA, Y., FUKUDA, S., ENDO, T. A., NAKATO, G., TAKAHASHI, D., NAKANISHI, Y., UETAKE, C., KATO, K., KATO, T., TAKAHASHI, M., FUKUDA, N. N., MURAKAMI, S., MIYAUCHI, E., HINO, S., ATARASHI, K., ONAWA, S., FUJIMURA, Y., LOCKETT, T., CLARKE, J. M., TOPPING, D. L., TOMITA, M., HORI, S., OHARA, O., MORITA, T., KOSEKI, H., KIKUCHI, J., HONDA, K., HASE, K. & OHNO, H. 2013. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature,* 504**,** 446-50.

GABAY, M., LI, Y. & FELSHER, D. W. 2014. MYC activation is a hallmark of cancer initiation and maintenance. *Cold Spring Harb Perspect Med,* 4.

GARCIA, J. M., SILVA, J. M., DOMINGUEZ, G., GONZALEZ, R., NAVARRO, A., CARRETERO, L., PROVENCIO, M., ESPANA, P. & BONILLA, F. 1999. Allelic loss of the PTEN region (10q23) in breast carcinomas of poor pathophenotype. *Breast Cancer Res Treat,* 57**,** 237-43.

GEUKING, M. B., KOLLER, Y., RUPP, S. & MCCOY, K. D. 2014. The interplay between the gut microbiota and the immune system. *Gut Microbes,* 5**,** 411-8.

GIARDINA, C., NAKANISHI, M., KHAN, A., KURATNIK, A., XU, W., BRENNER, B. & ROSENBERG, D. W. 2015. Regulation of VDR Expression in Apc-Mutant Mice, Human Colon Cancers and Adenomas. *Cancer Prev Res (Phila),* 8**,** 387-99.

GOCHMAN, E., MAHAJNA, J., SHENZER, P., DAHAN, A., BLATT, A., ELYAKIM, R. & REZNICK, A. Z. 2012. The expression of iNOS and nitrotyrosine in colitis and colon cancer in humans. *Acta Histochem,* 114**,** 827-35.

GRAHAM, D., HUYNH, N. N., HAMILTON, C. A., BEATTIE, E., SMITH, R. A., COCHEME, H. M., MURPHY, M. P. & DOMINICZAK, A. F. 2009. Mitochondria-targeted antioxidant MitoQ10 improves endothelial function and attenuates cardiac hypertrophy. *Hypertension,* 54**,** 322-8.

GRETEN, F. R., ECKMANN, L., GRETEN, T. F., PARK, J. M., LI, Z. W., EGAN, L. J., KAGNOFF, M. F. & KARIN, M. 2004. IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell,* 118**,** 285-96.

GRIS, D., YE, Z., IOCCA, H. A., WEN, H., CRAVEN, R. R., GRIS, P., HUANG, M., SCHNEIDER, M., MILLER, S. D. & TING, J. P. 2010. NLRP3 plays a critical role in the development of experimental autoimmune encephalomyelitis by mediating Th1 and Th17 responses. *J Immunol,* 185**,** 974-81.

GRIVENNIKOV, S. 2013. Inflammation and colorectal cancer: colitis-associated neoplasia. *Seminars in Immunopathology,* 35**,** 229-244.

GRIVENNIKOV, S., KARIN, E., TERZIC, J., MUCIDA, D., YU, G. Y., VALLABHAPURAPU, S., SCHELLER, J., ROSE-JOHN, S., CHEROUTRE, H., ECKMANN, L. & KARIN, M. 2009. IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. *Cancer Cell,* 15**,** 103-13.

GRIVENNIKOV, S. I. & KARIN, M. 2010a. Dangerous liaisons: STAT3 and NF-kappaB collaboration and crosstalk in cancer. *Cytokine Growth Factor Rev,* 21**,** 11-9.

GRIVENNIKOV, S. I. & KARIN, M. 2010b. Inflammation and oncogenesis: a vicious connection. *Curr Opin Genet Dev,* 20**,** 65-71.

GRIVENNIKOV, S. I., WANG, K., MUCIDA, D., STEWART, C. A., SCHNABL, B., JAUCH, D., TANIGUCHI, K., YU, G. Y., OSTERREICHER, C. H., HUNG, K. E., DATZ, C., FENG, Y., FEARON, E. R., OUKKA, M., TESSAROLLO, L., COPPOLA, V., YAROVINSKY, F., CHEROUTRE, H., ECKMANN, L., TRINCHIERI, G. & KARIN, M. 2012. Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. *Nature,* 491**,** 254-8.

GROSS, O., POECK, H., BSCHEIDER, M., DOSTERT, C., HANNESSCHLAGER, N., ENDRES, S., HARTMANN, G., TARDIVEL, A., SCHWEIGHOFFER, E., TYBULEWICZ, V., MOCSAI, A., TSCHOPP, J. & RULAND, J. 2009. Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. *Nature,* 459**,** 433-6.

GROSS, O., YAZDI, A. S., THOMAS, C. J., MASIN, M., HEINZ, L. X., GUARDA, G., QUADRONI, M., DREXLER, S. K. & TSCHOPP, J. 2012. Inflammasome activators induce interleukin-1alpha secretion via distinct pathways with differential requirement for the protease function of caspase-1. *Immunity,* 36**,** 388-400.

GUIMBAUD, R., BERTRAND, V., CHAUVELOT-MOACHON, L., QUARTIER, G., VIDON, N., GIROUD, J. P., COUTURIER, D. & CHAUSSADE, S. 1998. Network of inflammatory cytokines and correlation with disease activity in ulcerative colitis. *Am J Gastroenterol,* 93**,** 2397-404.

GUO, H. T., CALLAWAY, J. B. & TING, J. P. Y. 2015a. Inflammasomes: mechanism of action, role in disease, and therapeutics. *Nature Medicine,* 21**,** 677-687.

GUO, W., HU, S., ELGEHAMA, A., SHAO, F., REN, R., LIU, W., ZHANG, W., WANG, X., TAN, R., XU, Q., SUN, Y. & JIAO, R. 2015b. Fumigaclavine C ameliorates dextran sulfate sodium-induced murine experimental colitis via NLRP3 inflammasome inhibition. *J Pharmacol Sci,* 129**,** 101-6.

GUO, W., LIU, W., JIN, B., GENG, J., LI, J., DING, H., WU, X., XU, Q., SUN, Y. & GAO, J. 2015c. Asiatic acid ameliorates dextran sulfate sodium-induced murine experimental colitis via suppressing mitochondria-mediated NLRP3 inflammasome activation. *Int Immunopharmacol,* 24**,** 232-8.

GUO, W., SUN, Y., LIU, W., WU, X., GUO, L., CAI, P., WU, X., WU, X., SHEN, Y., SHU, Y., GU, Y. & XU, Q. 2014. Small molecule-driven mitophagy-mediated NLRP3 inflammasome inhibition is responsible for the prevention of colitis-associated cancer. *Autophagy,* 10**,** 972-85.

GUPTA, R. A. & DUBOIS, R. N. 2001. Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. *Nat Rev Cancer,* 1**,** 11-21.

GURUNG, P., LUKENS, J. R. & KANNEGANTI, T. D. 2015. Mitochondria: diversity in the regulation of the NLRP3 inflammasome. *Trends Mol Med,* 21**,** 193-201.

HAGUE, A., MANNING, A. M., HANLON, K. A., HUSCHTSCHA, L. I., HART, D. & PARASKEVA, C. 1993. Sodium butyrate induces apoptosis in human colonic tumour cell lines in a p53-independent pathway: implications for the possible role of dietary fibre in the prevention of large-bowel cancer. *Int J Cancer,* 55**,** 498-505.

HALLE, A., HORNUNG, V., PETZOLD, G. C., STEWART, C. R., MONKS, B. G., REINHECKEL, T., FITZGERALD, K. A., LATZ, E., MOORE, K. J. & GOLENBOCK, D. T. 2008. The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. *Nat Immunol,* 9**,** 857-65.

HAMER, H. M., JONKERS, D., VENEMA, K., VANHOUTVIN, S., TROOST, F. J. & BRUMMER, R. J. 2008. Review article: the role of butyrate on colonic function. *Aliment Pharmacol Ther,* 27**,** 104-19.

HANAUER, S. B. 2006. Inflammatory bowel disease: Epidemiology, pathogenesis, and therapeutic opportunities. *Inflammatory Bowel Diseases,* 12**,** S3-S9.

HAYAKAWA, K., MATSUDA, F. & SHIMIZU, H. 2016. Metabolome analysis of Saccharomyces cerevisiae and optimization of culture medium for S-adenosyl-L-methionine production. *AMB Express,* 6**,** 38.

HAYES, M. P., WANG, J. & NORCROSS, M. A. 1995. Regulation of interleukin-12 expression in human monocytes: selective priming by interferon-gamma of lipopolysaccharide-inducible p35 and p40 genes. *Blood,* 86**,** 646-50.

HE, X., WEI, Z., WANG, J., KOU, J., LIU, W., FU, Y. & YANG, Z. 2016a. Alpinetin attenuates inflammatory responses by suppressing TLR4 and NLRP3 signaling pathways in DSS-induced acute colitis. *Sci Rep,* 6**,** 28370.

HE, Y., ZENG, M. Y., YANG, D. H., METRO, B. & NUNEZ, G. 2016b. NEK7 is an essential mediator of NLRP3 activation downstream of potassium efflux. *Nature,* 530**,** 354-+.

HEAZLEWOOD, C. K., COOK, M. C., ERI, R., PRICE, G. R., TAURO, S. B., TAUPIN, D., THORNTON, D. J., PNG, C. W., CROCKFORD, T. L., CORNALL, R. J., ADAMS, R., KATO, M., NELMS, K. A., HONG, N. A., FLORIN, T. H., GOODNOW, C. C. & MCGUCKIN, M. A. 2008. Aberrant mucin assembly in mice causes endoplasmic reticulum stress and spontaneous inflammation resembling ulcerative colitis. *PLoS Med,* 5**,** e54.

HEERDT, B. G., HOUSTON, M. A. & AUGENLICHT, L. H. 1994. Potentiation by specific short-chain fatty acids of differentiation and apoptosis in human colonic carcinoma cell lines. *Cancer Res,* 54**,** 3288-93.

HEID, M. E., KEYEL, P. A., KAMGA, C., SHIVA, S., WATKINS, S. C. & SALTER, R. D. 2013. Mitochondrial reactive oxygen species induces NLRP3-dependent lysosomal damage and inflammasome activation. *J Immunol,* 191**,** 5230-8.

HEIKKILA, K., EBRAHIM, S. & LAWLOR, D. A. 2008. Systematic review of the association between circulating interleukin-6 (IL-6) and cancer. *Eur J Cancer,* 44**,** 937-45.

HERCUS, T. R., THOMAS, D., GUTHRIDGE, M. A., EKERT, P. G., KING-SCOTT, J., PARKER, M. W. & LOPEZ, A. F. 2009. The granulocyte-macrophage colony-stimulating factor receptor: linking its structure to cell signaling and its role in disease. *Blood,* 114**,** 1289-98.

HERNANDEZ, J. M., FARMA, J. M., COPPOLA, D., HAKAM, A., FULP, W. J., CHEN, D. T., SIEGEL, E. M., YEATMAN, T. J. & SHIBATA, D. 2011. Expression of the antiapoptotic protein survivin in colon cancer. *Clin Colorectal Cancer,* 10**,** 188-93.

HIROTA, S. A., NG, J., LUENG, A., KHAJAH, M., PARHAR, K., LI, Y., LAM, V., POTENTIER, M. S., NG, K., BAWA, M., MCCAFFERTY, D. M., RIOUX, K. P., GHOSH, S., XAVIER, R. J., COLGAN, S. P., TSCHOPP, J., MURUVE, D., MACDONALD, J. A. & BECK, P. L. 2011. NLRP3 inflammasome plays a key role in the regulation of intestinal homeostasis. *Inflamm Bowel Dis,* 17**,** 1359-72.

HIRT, C., EPPENBERGER-CASTORI, S., SCONOCCHIA, G., IEZZI, G., TORNILLO, L., TERRACCIANO, L., SPAGNOLI, G. C. & DROESER, R. A. 2013. Colorectal carcinoma infiltration by myeloperoxidase-expressing neutrophil granulocytes is associated with favorable prognosis. *Oncoimmunology,* 2**,** e25990-e25990.

HOFFMAN, H. M., MUELLER, J. L., BROIDE, D. H., WANDERER, A. A. & KOLODNER, R. D. 2001. Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. *Nat Genet,* 29**,** 301-5.

HORNUNG, V., ABLASSER, A., CHARREL-DENNIS, M., BAUERNFEIND, F., HORVATH, G., CAFFREY, D. R., LATZ, E. & FITZGERALD, K. A. 2009. AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature,* 458**,** 514.

HORWOOD, N. J., UDAGAWA, N., ELLIOTT, J., GRAIL, D., OKAMURA, H., KURIMOTO, M., DUNN, A. R., MARTIN, T. & GILLESPIE, M. T. 1998. Interleukin 18 inhibits osteoclast formation via T cell production of granulocyte macrophage colony-stimulating factor. *The Journal of clinical investigation,* 101**,** 595-603.

HU, B., ELINAV, E. & FLAVELL, R. A. 2011. Inflammasome-mediated suppression of inflammation-induced colorectal cancer progression is mediated by direct regulation of epithelial cell proliferation. *Cell Cycle,* 10**,** 1936-1939.

HU, B., ELINAV, E., HUBER, S., STROWIG, T., HAO, L. M., HAFEMANN, A., JIN, C. C., EISENBARTH, S. C. & FLAVELL, R. A. 2013. Microbiota-induced activation of epithelial IL-6 signaling links inflammasome-driven inflammation with transmissible cancer. *Proceedings of the National Academy of Sciences of the United States of America,* 110**,** 9862-9867.

HUANG, Y. J., QI, W. X., HE, A. N., SUN, Y. J., SHEN, Z. & YAO, Y. 2013. The prognostic value of survivin expression in patients with colorectal carcinoma: a meta-analysis. *Jpn J Clin Oncol,* 43**,** 988-95.

HUBER, M. A., KRAUT, N. & BEUG, H. 2005. Molecular requirements for epithelial–mesenchymal transition during tumor progression. *Current Opinion in Cell Biology,* 17**,** 548-558.

HUBER, S., GAGLIANI, N. & FLAVELL, R. A. 2012. Life, death, and miracles: Th17 cells in the intestine. *Eur J Immunol,* 42**,** 2238-45.

HUSON, D. H. & MITRA, S. 2012. Introduction to the analysis of environmental sequences: metagenomics with MEGAN. *Evolutionary Genomics.* Springer.

HUSSAIN, S. P., AMSTAD, P., RAJA, K., AMBS, S., NAGASHIMA, M., BENNETT, W. P., SHIELDS, P. G., HAM, A. J., SWENBERG, J. A., MARROGI, A. J. & HARRIS, C. C. 2000. Increased p53 mutation

load in noncancerous colon tissue from ulcerative colitis: a cancer-prone chronic inflammatory disease. *Cancer Res,* 60**,** 3333-7.

HUSSAIN, S. P., HOFSETH, L. J. & HARRIS, C. C. 2003. Radical causes of cancer. *Nat Rev Cancer,* 3**,** 276-85.

HUYCKE, M. M. & GASKINS, H. R. 2004. Commensal bacteria, redox stress, and colorectal cancer: mechanisms and models. *Exp Biol Med (Maywood),* 229**,** 586-97.

HYUN, Y. S., HAN, D. S., LEE, A. R., EUN, C. S., YOUN, J. & KIM, H. Y. 2012. Role of IL-17A in the development of colitis-associated cancer. *Carcinogenesis,* 33**,** 931-6.

IGNACIO, A., MORALES, C. I., CAMARA, N. O. S. & ALMEIDA, R. R. 2016. Innate Sensing of the Gut Microbiota: Modulation of Inflammatory and Autoimmune Diseases. *Frontiers in Immunology,* 7.

ISCHIROPOULOS, H., ZHU, L., CHEN, J., TSAI, M., MARTIN, J. C., SMITH, C. D. & BECKMAN, J. S. 1992. Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. *Arch Biochem Biophys,* 298**,** 431-7.

ISHIKAWA, T. O. & HERSCHMAN, H. R. 2010. Tumor formation in a mouse model of colitis-associated colon cancer does not require COX-1 or COX-2 expression. *Carcinogenesis,* 31**,** 729-36.

ITANI, S., WATANABE, T., NADATANI, Y., SUGIMURA, N., SHIMADA, S., TAKEDA, S., OTANI, K., HOSOMI, S., NAGAMI, Y., TANAKA, F., KAMATA, N., YAMAGAMI, H., TANIGAWA, T., SHIBA, M., TOMINAGA, K., FUJIWARA, Y. & ARAKAWA, T. 2016. NLRP3 inflammasome has a protective effect against oxazolone-induced colitis: a possible role in ulcerative colitis. *Scientific Reports,* 6**,** 39075.

IVANOV, II, ATARASHI, K., MANEL, N., BRODIE, E. L., SHIMA, T., KARAOZ, U., WEI, D., GOLDFARB, K. C., SANTEE, C. A., LYNCH, S. V., TANOUE, T., IMAOKA, A., ITOH, K., TAKEDA, K., UMESAKI, Y., HONDA, K. & LITTMAN, D. R. 2009. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell,* 139**,** 485-98.

JACKSON, A. L. & LOEB, L. A. 2001. The contribution of endogenous sources of DNA damage to the multiple mutations in cancer. *Mutat Res,* 477**,** 7-21.

JANOWSKI, A. M., KOLB, R., ZHANG, W. & SUTTERWALA, F. S. 2013. Beneficial and Detrimental Roles of NLRs in Carcinogenesis. *Front Immunol,* 4**,** 370.

JI, L., WEI, Y., JIANG, T. & WANG, S. 2014. Correlation of Nrf2, NQO1, MRP1, cmyc and p53 in colorectal cancer and their relationships to clinicopathologic features and survival. *International journal of clinical and experimental pathology,* 7**,** 1124-1131.

JIANG, H., HE, H., CHEN, Y., HUANG, W., CHENG, J., YE, J., WANG, A., TAO, J., WANG, C., LIU, Q., JIN, T., JIANG, W., DENG, X. & ZHOU, R. 2017. Identification of a selective and direct NLRP3 inhibitor to treat inflammatory disorders. *The Journal of Experimental Medicine*.

JIANG, X., ZHONG, L., SUN, D. & RONG, L. 2016. Magnesium lithospermate B acts against dextran sodiumsulfate-induced ulcerative colitis by inhibiting activation of the NRLP3/ASC/Caspase-1 pathway. *Environ Toxicol Pharmacol,* 41**,** 72-7.

JOBIN, C. 2012. Colorectal cancer: CRC--all about microbial products and barrier function? *Nat Rev Gastroenterol Hepatol,* 9**,** 694-6.

JOHANSSON, M. E., PHILLIPSON, M., PETERSSON, J., VELCICH, A., HOLM, L. & HANSSON, G. C. 2008. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc Natl Acad Sci U S A,* 105**,** 15064-9.

KALER, P., AUGENLICHT, L. & KLAMPFER, L. 2009a. Macrophage-derived IL-1beta stimulates Wnt signaling and growth of colon cancer cells: a crosstalk interrupted by vitamin D3. *Oncogene,* 28**,** 3892-902.

KALER, P., GODASI, B. N., AUGENLICHT, L. & KLAMPFER, L. 2009b. The NF-kappaB/AKT-dependent Induction of Wnt Signaling in Colon Cancer Cells by Macrophages and IL-1beta. *Cancer Microenviron,* 2**,** 69-80.

KAMADA, N., SEO, S. U., CHEN, G. Y. & NUNEZ, G. 2013. Role of the gut microbiota in immunity and inflammatory disease. *Nat Rev Immunol,* 13**,** 321-35.

KANAI, T., KAMADA, N. & HISAMATSU, T. 2013. Clinical strategies for the blockade of IL-18 in inflammatory bowel diseases. *Curr Drug Targets,* 14**,** 1392-9.

KAPLAN, G. G. 2015. The global burden of IBD: from 2015 to 2025. *Nature Reviews Gastroenterology &Amp; Hepatology,* 12**,** 720.

KARIN, M. & GRETEN, F. R. 2005. NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol,* 5**,** 749-59.

KARKI, R., MALIREDDI, R. K. S., ZHU, Q. F. & KANNEGANTI, T. D. 2017. NLRC3 regulates cellular proliferation and apoptosis to attenuate the development of colorectal cancer. *Cell Cycle,* 16**,** 1243-1251.

KARKI, R., MAN, S. M., MALIREDDI, R. K., KESAVARDHANA, S., ZHU, Q., BURTON, A. R., SHARMA, B. R., QI, X., PELLETIER, S., VOGEL, P., ROSENSTIEL, P. & KANNEGANTI, T. D. 2016. NLRC3 is an inhibitory sensor of PI3K-mTOR pathways in cancer. *Nature*.

KARPE, A. V., BEALE, D. J., GODHANI, N. B., MORRISON, P. D., HARDING, I. H. & PALOMBO, E. A. 2015. Untargeted Metabolic Profiling of Winery-Derived Biomass Waste Degradation by Penicillium chrysogenum. *J Agric Food Chem,* 63**,** 10696-704.

KASDAGLY, M., RADHAKRISHNAN, S., REDDIVARI, L., VEERAMACHANENI, D. N. & VANAMALA, J. 2014. Colon carcinogenesis: influence of Western diet-induced obesity and targeting stem cells using dietary bioactive compounds. *Nutrition,* 30**,** 1242-56.

KAYAGAKI, N., WARMING, S., LAMKANFI, M., VANDE WALLE, L., LOUIE, S., DONG, J., NEWTON, K., QU, Y., LIU, J., HELDENS, S., ZHANG, J., LEE, W. P., ROOSE-GIRMA, M. & DIXIT, V. M. 2011. Non-canonical inflammasome activation targets caspase-11. *Nature,* 479**,** 117-21.

KE, P., SHAO, B. Z., XU, Z. Q., WEI, W., HAN, B. Z., CHEN, X. W., SU, D. F. & LIU, C. 2016. Activation of Cannabinoid Receptor 2 Ameliorates DSS-Induced Colitis through Inhibiting NLRP3 Inflammasome in Macrophages. *PLoS One,* 11**,** e0155076.

KELLY, C. J., ZHENG, L., CAMPBELL, E. L., SAEEDI, B., SCHOLZ, C. C., BAYLESS, A. J., WILSON, K. E., GLOVER, L. E., KOMINSKY, D. J., MAGNUSON, A., WEIR, T. L., EHRENTRAUT, S. F., PICKEL, C., KUHN, K. A., LANIS, J. M., NGUYEN, V., TAYLOR, C. T. & COLGAN, S. P. 2015. Crosstalk between Microbiota-Derived Short-Chain Fatty Acids and Intestinal Epithelial HIF Augments Tissue Barrier Function. *Cell Host Microbe,* 17**,** 662-71.

KELSEN, J. & BALDASSANO, R. N. 2008. Inflammatory bowel disease: the difference between children and adults. *Inflamm Bowel Dis,* 14 Suppl 2**,** S9-11.

KESHAVARZIAN, A., BANAN, A., FARHADI, A., KOMANDURI, S., MUTLU, E., ZHANG, Y. & FIELDS, J. Z. 2003. Increases in free radicals and cytoskeletal protein oxidation and nitration in the colon of patients with inflammatory bowel disease. *Gut,* 52**,** 720-8.

KIM, P. J., PLESCIA, J., CLEVERS, H., FEARON, E. R. & ALTIERI, D. C. 2003. Survivin and molecular pathogenesis of colorectal cancer. *The Lancet,* 362**,** 205-209.

KIM, S. C., TONKONOGY, S. L., KARRASCH, T., JOBIN, C. & SARTOR, R. B. 2007. Dual-association of gnotobiotic IL-10-/- mice with 2 nonpathogenic commensal bacteria induces aggressive pancolitis. *Inflamm Bowel Dis,* 13**,** 1457-66.

KITAJIMA, S., MORIMOTO, M., SAGARA, E., SHIMIZU, C. & IKEDA, Y. 2001. Dextran sodium sulfate-induced colitis in germ-free IQI/Jic mice. *Exp Anim,* 50**,** 387-95.

KLIONSKY, D. J., ABDALLA, F. C., ABELIOVICH, H., ABRAHAM, R. T., ACEVEDO-AROZENA, A., ADELI, K., AGHOLME, L., AGNELLO, M., AGOSTINIS, P., AGUIRRE-GHISO, J. A., AHN, H. J., AIT-MOHAMED, O., AIT-SI-ALI, S., AKEMATSU, T., AKIRA, S., AL-YOUNES, H. M., AL-ZEER, M. A., ALBERT, M. L., ALBIN, R. L., ALEGRE-ABARRATEGUI, J., ALEO, M. F., ALIREZAEI, M., ALMASAN, A., ALMONTE-BECERRIL, M., AMANO, A., AMARAVADI, R. K., AMARNATH, S., AMER, A. O., ANDRIEU-ABADIE, N., ANANTHARAM, V., ANN, D. K., ANOOPKUMAR-DUKIE, S., AOKI, H., APOSTOLOVA, N., ARANCIA, G., ARIS, J. P., ASANUMA, K., ASARE, N. Y. O., ASHIDA, H., ASKANAS, V., ASKEW, D. S., AUBERGER, P., BABA, M., BACKUES, S. K., BAEHRECKE, E. H., BAHR, B. A., BAI, X.-Y., BAILLY, Y., BAIOCCHI, R., BALDINI, G., BALDUINI, W., BALLABIO, A., BAMBER, B. A., BAMPTON, E. T. W., JUHÁSZ, G., BARTHOLOMEW, C. R., BASSHAM, D. C., BAST, R. C., BATOKO, H., BAY, B.-H., BEAU, I., BÉCHET, D. M., BEGLEY, T. J., BEHL, C., BEHRENDS, C., BEKRI, S., BELLAIRE, B., BENDALL, L. J., BENETTI, L., BERLIOCCHI, L., BERNARDI, H., BERNASSOLA, F., BESTEIRO, S., BHATIA-KISSOVA, I., BI, X., BIARD-PIECHACZYK, M., BLUM, J. S., BOISE, L. H., BONALDO, P., BOONE, D. L., BORNHAUSER, B. C., BORTOLUCI, K. R., BOSSIS, I., BOST, F., BOURQUIN, J.-P., BOYA, P., BOYER-GUITTAUT, M., BOZHKOV, P. V., BRADY, N. R., BRANCOLINI, C., BRECH, A., BRENMAN, J. E., BRENNAND, A., BRESNICK, E. H., BREST, P., BRIDGES, D., BRISTOL, M. L., BROOKES, P. S., BROWN, E. J., BRUMELL, J. H., et al. 2012. Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy,* 8**,** 445-544.

KMIEC, Z., CYMAN, M. & SLEBIODA, T. J. 2017. Cells of the innate and adaptive immunity and their interactions in inflammatory bowel disease. *Adv Med Sci,* 62**,** 1-16.

KOBAYASHI, K. S., CHAMAILLARD, M., OGURA, Y., HENEGARIU, O., INOHARA, N., NUNEZ, G. & FLAVELL, R. A. 2005. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science,* 307**,** 731-4.

KOH, A., DE VADDER, F., KOVATCHEVA-DATCHARY, P. & BACKHED, F. 2016. From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites. *Cell,* 165**,** 1332-1345.

KOMODA, H., TANAKA, Y., HONDA, M., MATSUO, Y., HAZAMA, K. & TAKAO, T. 1998. Interleukin-6 levels in colorectal cancer tissues. *World J Surg,* 22**,** 895-8.

KONDO, S., TOYOKUNI, S., TSURUYAMA, T., OZEKI, M., TACHIBANA, T., ECHIZENYA, M., HIAI, H., ONODERA, H. & IMAMURA, M. 2002. Peroxynitrite-mediated stress is associated with proliferation of human metastatic colorectal carcinoma in the liver. *Cancer Lett,* 179**,** 87-93.

KOSTIC, A. D., GEVERS, D., PEDAMALLU, C. S., MICHAUD, M., DUKE, F., EARL, A. M., OJESINA, A. I., JUNG, J., BASS, A. J., TABERNERO, J., BASELGA, J., LIU, C., SHIVDASANI, R. A., OGINO, S., BIRREN, B. W., HUTTENHOWER, C., GARRETT, W. S. & MEYERSON, M. 2012. Genomic

analysis identifies association of Fusobacterium with colorectal carcinoma. *Genome Res,* 22**,** 292-8.

KRAWISZ, J. E., SHARON, P. & STENSON, W. F. 1984. Quantitative assay for acute intestinal inflammation based on myeloperoxidase activity. Assessment of inflammation in rat and hamster models. *Gastroenterology,* 87**,** 1344-50.

KRELIN, Y., VORONOV, E., DOTAN, S., ELKABETS, M., REICH, E., FOGEL, M., HUSZAR, M., IWAKURA, Y., SEGAL, S., DINARELLO, C. A. & APTE, R. N. 2007. Interleukin-1beta-driven inflammation promotes the development and invasiveness of chemical carcinogen-induced tumors. *Cancer Res,* 67**,** 1062-71.

KRISHNAN, S. M., DOWLING, J. K., LING, Y. H., DIEP, H., CHAN, C. T., FERENS, D., KETT, M. M., PINAR, A., SAMUEL, C. S., VINH, A., ARUMUGAM, T. V., HEWITSON, T. D., KEMP-HARPER, B. K., ROBERTSON, A. A. B., COOPER, M. A., LATZ, E., MANSELL, A., SOBEY, C. G. & DRUMMOND, G. R. 2016. Inflammasome activity is essential for one kidney/deoxycorticosterone acetate/salt-induced hypertension in mice. *British Journal of Pharmacology,* 173**,** 752-765.

KUCZYNSKI, J., STOMBAUGH, J., WALTERS, W. A., GONZÁLEZ, A., CAPORASO, J. G. & KNIGHT, R. 2012. Using QIIME to analyze 16S rRNA gene sequences from microbial communities. *Current protocols in microbiology,* 27**,** 1E. 5.1-1E. 5.20.

KULLBERG, M. C., WARD, J. M., GORELICK, P. L., CASPAR, P., HIENY, S., CHEEVER, A., JANKOVIC, D. & SHER, A. 1998. Helicobacter hepaticus triggers colitis in specific-pathogen-free interleukin-10 (IL-10)-deficient mice through an IL-12- and gamma interferon-dependent mechanism. *Infect Immun,* 66**,** 5157-66.

KUMMER, J. A., BROEKHUIZEN, R., EVERETT, H., AGOSTINI, L., KUIJK, L., MARTINON, F., VAN BRUGGEN, R. & TSCHOPP, J. 2007. Inflammasome components NALP 1 and 3 show distinct but separate expression profiles in human tissues suggesting a site-specific role in the inflammatory response. *J Histochem Cytochem,* 55**,** 443-52.

LALIBERTE, R. E., PERREGAUX, D. G., HOTH, L. R., ROSNER, P. J., JORDAN, C. K., PEESE, K. M., EGGLER, J. F., DOMBROSKI, M. A., GEOGHEGAN, K. F. & GABEL, C. A. 2003. Glutathione s-transferase omega 1-1 is a target of cytokine release inhibitory drugs and may be responsible for their effect on interleukin-1beta posttranslational processing. *J Biol Chem,* 278**,** 16567-78.

LAMKANFI, M., MUELLER, J. L., VITARI, A. C., MISAGHI, S., FEDOROVA, A., DESHAYES, K., LEE, W. P., HOFFMAN, H. M. & DIXIT, V. M. 2009. Glyburide inhibits the Cryopyrin/Nalp3 inflammasome. *Journal of Cell Biology,* 187**,** 61-70.

LANGILLE, M. G., MEEHAN, C. J., KOENIG, J. E., DHANANI, A. S., ROSE, R. A., HOWLETT, S. E. & BEIKO, R. G. 2014. Microbial shifts in the aging mouse gut. *Microbiome,* 2**,** 50.

LAPHAM, A., ADAMS, J. E., PATERSON, A., LEE, M., BRIMMELL, M. & PACKHAM, G. 2009. The Bcl-w promoter is activated by β-catenin/TCF4 in human colorectal carcinoma cells. *Gene,* 432**,** 112-117.

LATZ, E., XIAO, T. S. & STUTZ, A. 2013. Activation and regulation of the inflammasomes. *Nat Rev Immunol,* 13**,** 397-411.

LEAN, Q. Y., ERI, R. D., RANDALL-DEMLLO, S., SOHAL, S. S., STEWART, N., PETERSON, G. M., GUEVEN, N. & PATEL, R. P. 2015. Orally Administered Enoxaparin Ameliorates Acute Colitis by Reducing Macrophage-Associated Inflammatory Responses. *Plos One,* 10.

LECLEIRE, S., HASSAN, A., MARION-LETELLIER, R., ANTONIETTI, M., SAVOYE, G., BOLE-FEYSOT, C., LEREBOURS, E., DUCROTTE, P., DECHELOTTE, P. & COEFFIER, M. 2008. Combined glutamine and arginine decrease proinflammatory cytokine production by biopsies from Crohn's patients in association with changes in nuclear factor-kappaB and p38 mitogen-activated protein kinase pathways. *J Nutr,* 138**,** 2481-6.

LEE, G., GORETSKY, T., MANAGLIA, E., DIRISINA, R., SINGH, A. P., BROWN, J. B., MAY, R., YANG, G. Y., RAGHEB, J. W., EVERS, B. M., WEBER, C. R., TURNER, J. R., HE, X. C., KATZMAN, R. B., LI, L. & BARRETT, T. A. 2010. Phosphoinositide 3-kinase signaling mediates beta-catenin activation in intestinal epithelial stem and progenitor cells in colitis. *Gastroenterology,* 139**,** 869-81, 881.e1-9.

LEE, K. S., KWAK, Y., NAM, K. H., KIM, D.-W., KANG, S.-B., CHOE, G., KIM, W. H. & LEE, H. S. 2016. Favorable prognosis in colorectal cancer patients with co-expression of c-MYC and ß-catenin. *BMC cancer,* 16**,** 730-730.

LEEMANS, J. C., CASSEL, S. L. & SUTTERWALA, F. S. 2011. Sensing damage by the NLRP3 inflammasome. *Immunol Rev,* 243**,** 152-62.

LEVINE, J. S. & BURAKOFF, R. 2011. Extraintestinal Manifestations of Inflammatory Bowel Disease. *Gastroenterology & Hepatology,* 7**,** 235-241.

LI, F., ACKERMANN, E. J., BENNETT, C. F., ROTHERMEL, A. L., PLESCIA, J., TOGNIN, S., VILLA, A., MARCHISIO, P. C. & ALTIERI, D. C. 1999. Pleiotropic cell-division defects and apoptosis induced by interference with survivin function. *Nat Cell Biol,* 1**,** 461-6.

LI, H. J., REINHARDT, F., HERSCHMAN, H. R. & WEINBERG, R. A. 2012. Cancer-stimulated mesenchymal stem cells create a carcinoma stem cell niche via prostaglandin E2 signaling. *Cancer Discov,* 2**,** 840-55.

LI, L. T., JIANG, G., CHEN, Q. & ZHENG, J. N. 2015. Ki67 is a promising molecular target in the diagnosis of cancer (review). *Mol Med Rep,* 11**,** 1566-72.

LI, W., KHOR, T. O., XU, C., SHEN, G., JEONG, W. S., YU, S. & KONG, A. N. 2008. Activation of Nrf2-antioxidant signaling attenuates NFkappaB-inflammatory response and elicits apoptosis. *Biochem Pharmacol,* 76**,** 1485-9.

LI, Y., WANG, X., YUE, P., TAO, H., RAMALINGAM, S. S., OWONIKOKO, T. K., DENG, X., WANG, Y., FU, H., KHURI, F. R. & SUN, S. Y. 2013. Protein phosphatase 2A and DNA-dependent protein kinase are involved in mediating rapamycin-induced Akt phosphorylation. *J Biol Chem,* 288**,** 13215-24.

LIGUMSKY, M., SIMON, P. L., KARMELI, F. & RACHMILEWITZ, D. 1990. Role of interleukin 1 in inflammatory bowel disease--enhanced production during active disease. *Gut,* 31**,** 686-9.

LIN, M. S., HUANG, J. X., CHEN, W. C., ZHANG, B. F., FANG, J., ZHOU, Q., HU, Y. & GAO, H. J. 2011. Expression of PPARgamma and PTEN in human colorectal cancer: An immunohistochemical study using tissue microarray methodology. *Oncol Lett,* 2**,** 1219-1224.

LIU, J., DUAN, Y., CHENG, X., CHEN, X., XIE, W., LONG, H., LIN, Z. & ZHU, B. 2011. IL-17 is associated with poor prognosis and promotes angiogenesis via stimulating VEGF production of cancer cells in colorectal carcinoma. *Biochem Biophys Res Commun,* 407**,** 348-54.

LIU, L., DONG, Y., YE, M., JIN, S., YANG, J., JOOSSE, M. E., SUN, Y., ZHANG, J., LAZAREV, M., BRANT, S. R., SAFAR, B., MAROHN, M., MEZEY, E. & LI, X. 2016a. The Pathogenic Role of NLRP3

Inflammasome Activation in Inflammatory Bowel Diseases of Both Mice and Humans. *J Crohns Colitis*.

LIU, R. R., TRUAX, A. D., CHEN, L., HU, P. Z., LI, Z. S., CHEN, J., SONG, C. J., CHEN, L. H. & TING, J. P. Y. 2015. Expression profile of innate immune receptors, NLRs and AIM2, in human colorectal cancer: correlation with cancer stages and inflammasome components. *Oncotarget,* 6**,** 33456-33469.

LIU, W., GUO, W., HANG, N., YANG, Y., WU, X., SHEN, Y., CAO, J., SUN, Y. & XU, Q. 2016b. MALT1 inhibitors prevent the development of DSS-induced experimental colitis in mice via inhibiting NF-kappaB and NLRP3 inflammasome activation. *Oncotarget,* 7**,** 30536-49.

LIU, W., GUO, W., WU, J., LUO, Q., TAO, F., GU, Y., SHEN, Y., LI, J., TAN, R., XU, Q. & SUN, Y. 2013. A novel benzo[d]imidazole derivate prevents the development of dextran sulfate sodium-induced murine experimental colitis via inhibition of NLRP3 inflammasome. *Biochem Pharmacol,* 85**,** 1504-12.

LIU, X., ZHOU, W., ZHANG, X., LU, P., DU, Q., TAO, L., DING, Y., WANG, Y. & HU, R. 2016c. Dimethyl fumarate ameliorates dextran sulfate sodium-induced murine experimental colitis by activating Nrf2 and suppressing NLRP3 inflammasome activation. *Biochem Pharmacol,* 112**,** 37-49.

LOFTUS, E. V., JR. 2004. Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences. *Gastroenterology,* 126**,** 1504-17.

LOHER, F., BAUER, C., LANDAUER, N., SCHMALL, K., SIEGMUND, B., LEHR, H. A., DAUER, M., SCHOENHARTING, M., ENDRES, S. & EIGLER, A. 2004. The interleukin-1 beta-converting enzyme inhibitor pralnacasan reduces dextran sulfate sodium-induced murine colitis and T helper 1 T-cell activation. *J Pharmacol Exp Ther,* 308**,** 583-90.

LOVING, C. L., OSORIO, M., KIM, Y. G., NUNEZ, G., HUGHES, M. A. & MERKEL, T. J. 2009. Nod1/Nod2-mediated recognition plays a critical role in induction of adaptive immunity to anthrax after aerosol exposure. *Infect Immun,* 77**,** 4529-37.

LU, D., HUANG, J. & BASU, A. 2006. Protein kinase Cepsilon activates protein kinase B/Akt via DNA-PK to protect against tumor necrosis factor-alpha-induced cell death. *J Biol Chem,* 281**,** 22799-807.

LUDWIG-PORTUGALL, I., BARTOK, E., DHANA, E., EVERS, B. D. G., PRIMIANO, M. J., HALL, J. P., FRANKLIN, B. S., KNOLLE, P. A., HORNUNG, V., HARTMANN, G., BOOR, P., LATZ, E. & KURTS, C. 2016. An NLRP3-specific inflammasome inhibitor attenuates crystal-induced kidney fibrosis in mice. *Kidney International,* 90**,** 525-539.

LUSCHER, B. 2012. MAD1 and its life as a MYC antagonist: an update. *Eur J Cell Biol,* 91**,** 506-14.

MACIA, L., TAN, J., VIEIRA, A. T., LEACH, K., STANLEY, D., LUONG, S., MARUYA, M., MCKENZIE, C. I., HIJIKATA, A., WONG, C., BINGE, L., THORBURN, A. N., CHEVALIER, N., ANG, C., MARINO, E., ROBERT, R., OFFERMANNS, S., TEIXEIRA, M. M., MOORE, R. J., FLAVELL, R. A., FAGARASAN, S. & MACKAY, C. R. 2015. Metabolite-sensing receptors GPR43 and GPR109A facilitate dietary fibre-induced gut homeostasis through regulation of the inflammasome. *Nature Communications,* 6.

MALIK, A., SHARMA, D., ZHU, Q., KARKI, R., GUY, C. S., VOGEL, P. & KANNEGANTI, T.-D. 2016. IL-33 regulates the IgA-microbiota axis to restrain IL-1α–dependent colitis and tumorigenesis. *The Journal of Clinical Investigation,* 126**,** 4469-4481.

MALOY, K. J. & POWRIE, F. 2011. Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature,* 474**,** 298-306.

MAN, S. M., KARKI, R. & KANNEGANTI, T. D. 2017. Molecular mechanisms and functions of pyroptosis, inflammatory caspases and inflammasomes in infectious diseases. *Immunol Rev,* 277**,** 61-75.

MANGAN, M. S. J., OLHAVA, E. J., ROUSH, W. R., SEIDEL, H. M., GLICK, G. D. & LATZ, E. 2018. Targeting the NLRP3 inflammasome in inflammatory diseases. *Nat Rev Drug Discov*.

MARIATHASAN, S., WEISS, D. S., NEWTON, K., MCBRIDE, J., O'ROURKE, K., ROOSE-GIRMA, M., LEE, W. P., WEINRAUCH, Y., MONACK, D. M. & DIXIT, V. M. 2006. Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature,* 440**,** 228-32.

MARION-LETELLIER, R., SAVOYE, G. & GHOSH, S. 2016. IBD: In Food We Trust. *J Crohns Colitis,* 10**,** 1351-1361.

MARKS, D. J. B. & SEGAL, A. W. 2008. Innate immunity in inflammatory bowel disease: a disease hypothesis. *The Journal of pathology,* 214**,** 260-266.

MARQUEZ-FLORES, Y. K., VILLEGAS, I., CARDENO, A., ROSILLO, M. A. & ALARCON-DE-LA-LASTRA, C. 2016. Apigenin supplementation protects the development of dextran sulfate sodium-induced murine experimental colitis by inhibiting canonical and non-canonical inflammasome signaling pathways. *J Nutr Biochem,* 30**,** 143-52.

MARTINON, F., BURNS, K. & TSCHOPP, J. 2002. The inflammasome: A molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Molecular Cell,* 10**,** 417-426.

MARTINON, F., MAYOR, A. & TSCHOPP, J. 2009. The Inflammasomes: Guardians of the Body. *Annual Review of Immunology,* 27**,** 229-265.

MARTINON, F., PETRILLI, V., MAYOR, A., TARDIVEL, A. & TSCHOPP, J. 2006. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature,* 440**,** 237-41.

MASTERS, S. L., DUNNE, A., SUBRAMANIAN, S. L., HULL, R. L., TANNAHILL, G. M., SHARP, F. A., BECKER, C., FRANCHI, L., YOSHIHARA, E., CHEN, Z., MULLOOLY, N., MIELKE, L. A., HARRIS, J., COLL, R. C., MILLS, K. H., MOK, K. H., NEWSHOLME, P., NUNEZ, G., YODOI, J., KAHN, S. E., LAVELLE, E. C. & O'NEILL, L. A. 2010. Activation of the NLRP3 inflammasome by islet amyloid polypeptide provides a mechanism for enhanced IL-1beta in type 2 diabetes. *Nat Immunol,* 11**,** 897-904.

MAXWELL, J. R., YADAV, R., ROSSI, R. J., RUBY, C. E., WEINBERG, A. D., AGUILA, H. L. & VELLA, A. T. 2006. IL-18 bridges innate and adaptive immunity through IFN-gamma and the CD134 pathway. *J Immunol,* 177**,** 234-45.

MAZMANIAN, S. K., ROUND, J. L. & KASPER, D. L. 2008. A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature,* 453**,** 620-5.

MBODJI, K., CHARPENTIER, C., GUERIN, C., QUEREC, C., BOLE-FEYSOT, C., AZIZ, M., SAVOYE, G., DECHELOTTE, P. & MARION-LETELLIER, R. 2013. Adjunct therapy of n-3 fatty acids to 5-ASA ameliorates inflammatory score and decreases NF-kappaB in rats with TNBS-induced colitis. *J Nutr Biochem,* 24**,** 700-5.

MCALINDON, M. E., HAWKEY, C. J. & MAHIDA, Y. R. 1998. Expression of interleukin 1 beta and interleukin 1 beta converting enzyme by intestinal macrophages in health and inflammatory bowel disease. *Gut,* 42**,** 214-9.

MCGILLIGAN, V. E., GREGORY-KSANDER, M. S., LI, D., MOORE, J. E., HODGES, R. R., GILMORE, M. S., MOORE, T. C. & DARTT, D. A. 2013. Staphylococcus aureus activates the NLRP3 inflammasome in human and rat conjunctival goblet cells. *PLoS One,* 8**,** e74010.

MCGOVERN, D. & POWRIE, F. 2007. The IL23 axis plays a key role in the pathogenesis of IBD. *Gut,* 56**,** 1333-6.

MCGUCKIN, M. A., ERI, R. D., DAS, I., LOURIE, R. & FLORIN, T. H. 2011. Intestinal secretory cell ER stress and inflammation. *Biochem Soc Trans,* 39**,** 1081-5.

MCMILLAN, D. C., CANNA, K. & MCARDLE, C. S. 2003. Systemic inflammatory response predicts survival following curative resection of colorectal cancer. *Br J Surg,* 90**,** 215-9.

MEDZHITOV, R. & JANEWAY, C. A., JR. 2002. Decoding the patterns of self and nonself by the innate immune system. *Science,* 296**,** 298-300.

MELLING, N., KOWITZ, C. M., SIMON, R., BOKEMEYER, C., TERRACCIANO, L., SAUTER, G., IZBICKI, J. R. & MARX, A. H. 2016. High Ki67 expression is an independent good prognostic marker in colorectal cancer. *Journal of Clinical Pathology,* 69**,** 209.

MEMBERS, M. S. I. B., SANSONE, S.-A., FAN, T., GOODACRE, R., GRIFFIN, J. L., HARDY, N. W., KADDURAH-DAOUK, R., KRISTAL, B. S., LINDON, J., MENDES, P., MORRISON, N., NIKOLAU, B., ROBERTSON, D., SUMNER, L. W., TAYLOR, C., VAN DER WERF, M., VAN OMMEN, B. & FIEHN, O. 2007. The Metabolomics Standards Initiative. *Nature Biotechnology,* 25**,** 846.

MENG, G., ZHANG, F., FUSS, I., KITANI, A. & STROBER, W. 2009. A mutation in the Nlrp3 gene causing inflammasome hyperactivation potentiates Th17 cell-dominant immune responses. *Immunity,* 30**,** 860-74.

MERKLE, D., DOUGLAS, P., MOORHEAD, G. B., LEONENKO, Z., YU, Y., CRAMB, D., BAZETT-JONES, D. P. & LEES-MILLER, S. P. 2002. The DNA-dependent protein kinase interacts with DNA to form a protein-DNA complex that is disrupted by phosphorylation. *Biochemistry,* 41**,** 12706-14.

MIKAMI, K., NAITO, M., ISHIGURO, T., YANO, H., TOMIDA, A., YAMADA, T., TANAKA, N., SHIRAKUSA, T. & TSURUO, T. 1998. Immunological quantitation of DT-diaphorase in carcinoma cell lines and clinical colon cancers: advanced tumors express greater levels of DT-diaphorase. *Japanese journal of cancer research : Gann,* 89**,** 910-915.

MONTELEONE, G. & NEURATH, M. F. 2012 *Inflammatory bowel disease.*, Garland Science

MONTELEONE, G., TRAPASSO, F., PARRELLO, T., BIANCONE, L., STELLA, A., IULIANO, R., LUZZA, F., FUSCO, A. & PALLONE, F. 1999. Bioactive IL-18 expression is up-regulated in Crohn's disease. *J Immunol,* 163**,** 143-7.

MULDER, D. J., NOBLE, A. J., JUSTINICH, C. J. & DUFFIN, J. M. 2014. A tale of two diseases: The history of inflammatory bowel disease. *Journal of Crohns & Colitis,* 8**,** 341-348.

MUNKHOLM, P., LANGHOLZ, E., NIELSEN, O. H., KREINER, S. & BINDER, V. 1992. Incidence and prevalence of Crohn's disease in the county of Copenhagen, 1962-87: a sixfold increase in incidence. *Scand J Gastroenterol,* 27**,** 609-14.

NAIJA, N., KAROUI, S., SERGHINI, M., KALLEL, L., BOUBAKER, J. & FILALI, A. 2011. [Management of failure of infliximab in inflammatory bowel disease]. *Tunis Med,* 89**,** 517-21.

NALBANDIAN, A., KHAN, A. A., SRIVASTAVA, R., LLEWELLYN, K. J., TAN, B. C., SHUKR, N., FAZLI, Y., KIMONIS, V. E. & BENMOHAMED, L. 2017. Activation of the NLRP3 Inflammasome Is Associated with Valosin-Containing Protein Myopathy. *Inflammation,* 40**,** 21-41.

NARDIN, A. & ABASTADO, J. P. 2008. Macrophages and cancer. *Front Biosci,* 13**,** 3494-505.

NASIRI, S., KUENZIG, M. E. & BENCHIMOL, E. I. 2017. Long-term outcomes of pediatric inflammatory bowel disease. *Seminars in Pediatric Surgery,* 26**,** 398-404.

NEBIKER, C. A., HAN, J., EPPENBERGER-CASTORI, S., IEZZI, G., HIRT, C., AMICARELLA, F., CREMONESI, E., HUBER, X., PADOVAN, E., ANGRISANI, B., DROESER, R. A., ROSSO, R., BOLLI, M., OERTLI, D., VON HOLZEN, U., ADAMINA, M., MURARO, M. G., MENGUS, C., ZAJAC, P., SCONOCCHIA, G., ZUBER, M., TORNILLO, L., TERRACCIANO, L. & SPAGNOLI, G. C. 2014. GM-CSF Production by Tumor Cells Is Associated with Improved Survival in Colorectal Cancer. *Clin Cancer Res,* 20**,** 3094-106.

NEUDECKER, V., HANEKLAUS, M., JENSEN, O., KHAILOVA, L., MASTERSON, J. C., TYE, H., BIETTE, K., JEDLICKA, P., BRODSKY, K. S., GERICH, M. E., MACK, M., ROBERTSON, A. A. B., COOPER, M. A., FURUTA, G. T., DINARELLO, C. A., O'NEILL, L. A., ELTZSCHIG, H. K., MASTERS, S. L. & MCNAMEE, E. N. 2017. Myeloid-derived miR-223 regulates intestinal inflammation via repression of the NLRP3 inflammasome. *J Exp Med,* 214**,** 1737-1752.

NI, J., CHEN, S. F. & HOLLANDER, D. 1996. Effects of dextran sulphate sodium on intestinal epithelial cells and intestinal lymphocytes. *Gut,* 39**,** 234-41.

NIEUWDORP, M., GILIJAMSE, P. W., PAI, N. & KAPLAN, L. M. 2014. Role of the microbiome in energy regulation and metabolism. *Gastroenterology,* 146**,** 1525-33.

NING, C., LI, Y. Y., WANG, Y., HAN, G. C., WANG, R. X., XIAO, H., LI, X. Y., HOU, C. M., MA, Y. F., SHENG, D. S., SHEN, B. F., FENG, J. N., GUO, R. F., LI, Y. & CHEN, G. J. 2015. Complement activation promotes colitis-associated carcinogenesis through activating intestinal IL-1beta/IL-17A axis. *Mucosal Immunol,* 8**,** 1275-84.

NISTAL, E., FERNANDEZ-FERNANDEZ, N., VIVAS, S. & OLCOZ, J. L. 2015. Factors Determining Colorectal Cancer: The Role of the Intestinal Microbiota. *Front Oncol,* 5**,** 220.

NORMAND, S., DELANOYE-CRESPIN, A., BRESSENOT, A., HUOT, L., GRANDJEAN, T., PEYRIN-BIROULET, L., LEMOINE, Y., HOT, D. & CHAMAILLARD, M. 2011. Nod-like receptor pyrin domain-containing protein 6 (NLRP6) controls epithelial self-renewal and colorectal carcinogenesis upon injury. *Proceedings of the National Academy of Sciences,* 108**,** 9601.

NOVICK, D., KIM, S., KAPLANSKI, G. & DINARELLO, C. A. 2013. Interleukin-18, more than a Th1 cytokine. *Semin Immunol,* 25**,** 439-48.

NOWARSKI, R., JACKSON, R., GAGLIANI, N., DE ZOETE, M. R., PALM, N. W., BAILIS, W., LOW, J. S., HARMAN, C. C. D., GRAHAM, M., ELINAV, E. & FLAVELL, R. A. 2015. Epithelial IL-18 Equilibrium Controls Barrier Function in Colitis. *Cell,* 163**,** 1444-1456.

OANCEA, I., PNG, C. W., DAS, I., LOURIE, R., WINKLER, I. G., ERI, R., SUBRAMANIAM, N., JINNAH, H. A., MCWHINNEY, B. C., LEVESQUE, J. P., MCGUCKIN, M. A., DULEY, J. A. & FLORIN, T. H. J. 2013. A novel mouse model of veno-occlusive disease provides strategies to prevent thioguanine-induced hepatic toxicity. *Gut,* 62**,** 594-605.

OBAYA, A. J., MATEYAK, M. K. & SEDIVY, J. M. 1999. Mysterious liaisons: the relationship between c-Myc and the cell cycle. *Oncogene,* 18**,** 2934-41.

OBTUŁOWICZ, T., SWOBODA, M., SPEINA, E., GACKOWSKI, D., ROZALSKI, R., SIOMEK, A., JANIK, J., JANOWSKA, B., CIEŚLA, J. M., JAWIEN, A., BANASZKIEWICZ, Z., GUZ, J., DZIAMAN, T., SZPILA, A., OLINSKI, R. & TUDEK, B. 2010. Oxidative stress and 8-oxoguanine repair are enhanced in colon adenoma and carcinoma patients. *Mutagenesis,* 25**,** 463-471.

OKADA, M., MATSUZAWA, A., YOSHIMURA, A. & ICHIJO, H. 2014. The Lysosome Rupture-activated TAK1-JNK Pathway Regulates NLRP3 Inflammasome Activation. *Journal of Biological Chemistry,* 289.

OKAMURA, H., TSUTSUI, H., KOMATSU, T., YUTSUDO, M., HAKURA, A., TANIMOTO, T., TORIGOE, K., OKURA, T., NUKADA, Y., HATTORI, K., AKITA, K., NAMBA, M., TANABE, F., KONISHI, K., FUKUDA, S. & KURIMOTO, M. 1995. Cloning of a new cytokine that induces IFN-γ production by T cells. *Nature,* 378**,** 88.

OLSEN, J., KIRKEBY, L. T., OLSEN, J., EIHOLM, S., JESS, P., GOGENUR, I. & TROELSEN, J. T. 2015. High interleukin-6 mRNA expression is a predictor of relapse in colon cancer. *Anticancer Res,* 35**,** 2235-40.

OSHIO, H., ABE, T., ONOGAWA, T., OHTSUKA, H., SATO, T., II, T., FUKASE, K., MUTO, M., KATAYOSE, Y., OIKAWA, M., RIKIYAMA, T., EGAWA, S. & UNNO, M. 2008. Peroxisome proliferator-activated receptor alpha activates cyclooxygenase-2 gene transcription through bile acid transport in human colorectal cancer cell lines. *J Gastroenterol,* 43**,** 538-49.

OZAKI, E., CAMPBELL, M. & DOYLE, S. L. 2015. Targeting the NLRP3 inflammasome in chronic inflammatory diseases: current perspectives. *J Inflamm Res,* 8**,** 15-27.

PANDURANGAN, A. K., DIVYA, T., KUMAR, K., DINESHBABU, V., VELAVAN, B. & SUDHANDIRAN, G. 2018. Colorectal carcinogenesis: Insights into the cell death and signal transduction pathways: A review. *World J Gastrointest Oncol,* 10**,** 244-259.

PAPADAKIS, K. A. & TARGAN, S. R. 2000. Role of cytokines in the pathogenesis of inflammatory bowel disease. *Annu Rev Med,* 51**,** 289-98.

PARIAN, A. & LAZAREV, M. 2015. Who and how to screen for cancer in at-risk inflammatory bowel disease patients. *Expert Review of Gastroenterology & Hepatology,* 9**,** 731-746.

PATSOS, G., GERMANN, A., GEBERT, J. & DIHLMANN, S. 2010. Restoration of absent in melanoma 2 (AIM2) induces G2/M cell cycle arrest and promotes invasion of colorectal cancer cells. *Int J Cancer,* 126**,** 1838-1849.

PAULL, T. T., ROGAKOU, E. P., YAMAZAKI, V., KIRCHGESSNER, C. U., GELLERT, M. & BONNER, W. M. 2000. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. *Curr Biol,* 10**,** 886-95.

PELLEGRINI, C., ANTONIOLI, L., LOPEZ-CASTEJON, G., BLANDIZZI, C. & FORNAI, M. 2017. Canonical and Non-Canonical Activation of NLRP3 Inflammasome at the Crossroad between Immune Tolerance and Intestinal Inflammation. *Front Immunol,* 8**,** 36.

PENEAU, A., SAVOYE, G., TURCK, D., DAUCHET, L., FUMERY, M., SALLERON, J., LEREBOURS, E., LIGIER, K., VASSEUR, F., DUPAS, J. L., MOUTERDE, O., SPYCKERELLE, C., DJEDDI, D., PEYRIN-BIROULET, L., COLOMBEL, J. F. & GOWER-ROUSSEAU, C. 2013. Mortality and Cancer in Pediatric-Onset Inflammatory Bowel Disease: A Population-Based Study. *American Journal of Gastroenterology,* 108**,** 1647-1653.

PEPYS, M. B. & HIRSCHFIELD, G. M. 2003. C-reactive protein: a critical update. *J Clin Invest,* 111**,** 1805-12.

PERERA, A. P., FERNANDO, R., SHINDE, T., GUNDAMARAJU, R., SOUTHAM, B., SOHAL, S. S., ROBERTSON, A. A. B., SCHRODER, K., KUNDE, D. & ERI, R. 2018. MCC950, a specific small molecule inhibitor of NLRP3 inflammasome attenuates colonic inflammation in spontaneous colitis mice. *Scientific Reports,* 8**,** 8618.

PERERA, A. P., KUNDE, D. & ERI, R. 2017. NLRP3 Inhibitors as potential therapeutic agents for treatment of Inflammatory Bowel Disease. *Curr Pharm Des*.

PERREGAUX, D. & GABEL, C. A. 1994. Interleukin-1 beta maturation and release in response to ATP and nigericin. Evidence that potassium depletion mediated by these agents is a necessary and common feature of their activity. *J Biol Chem,* 269**,** 15195-203.

PERŠE, M. & CERAR, A. 2012. Dextran Sodium Sulphate Colitis Mouse Model: Traps and Tricks. *Journal of Biomedicine and Biotechnology,* 2012**,** 13.

PETERSSON, J., SCHREIBER, O., HANSSON, G. C., GENDLER, S. J., VELCICH, A., LUNDBERG, J. O., ROOS, S., HOLM, L. & PHILLIPSON, M. 2010. Importance and regulation of the colonic mucus barrier in a mouse model of colitis. *American Journal of Physiology-Gastrointestinal and Liver Physiology,* 300**,** G327-G333.

PETRILLI, V., PAPIN, S., DOSTERT, C., MAYOR, A., MARTINON, F. & TSCHOPP, J. 2007. Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell Death Differ,* 14**,** 1583-9.

PICKERT, G., NEUFERT, C., LEPPKES, M., ZHENG, Y., WITTKOPF, N., WARNTJEN, M., LEHR, H. A., HIRTH, S., WEIGMANN, B., WIRTZ, S., OUYANG, W., NEURATH, M. F. & BECKER, C. 2009. STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing. *J Exp Med,* 206**,** 1465-72.

PINAR, A., DOWLING, J. K., BITTO, N. J., ROBERTSON, A. A. B., LATZ, E., STEWART, C. R., DRUMMOND, G. R., COOPER, M. A., MCAULEY, J. L., TATE, M. D. & MANSELL, A. 2017. PB1-F2 Peptide Derived from Avian Influenza A Virus H7N9 Induces Inflammation via Activation of the NLRP3 Inflammasome. *Journal of Biological Chemistry,* 292**,** 826-+.

PIZARRO, T. T., MICHIE, M. H., BENTZ, M., WORARATANADHARM, J., SMITH, M. F., JR., FOLEY, E., MOSKALUK, C. A., BICKSTON, S. J. & COMINELLI, F. 1999. IL-18, a novel immunoregulatory cytokine, is up-regulated in Crohn's disease: expression and localization in intestinal mucosal cells. *J Immunol,* 162**,** 6829-35.

PONDER, A. & LONG, M. D. 2013. A clinical review of recent findings in the epidemiology of inflammatory bowel disease. *Clin Epidemiol,* 5**,** 237-47.

POPIVANOVA, B. K., KITAMURA, K., WU, Y., KONDO, T., KAGAYA, T., KANEKO, S., OSHIMA, M., FUJII, C. & MUKAIDA, N. 2008. Blocking TNF-alpha in mice reduces colorectal carcinogenesis associated with chronic colitis. *J Clin Invest,* 118**,** 560-70.

PORITZ, L. S., GARVER, K. I., GREEN, C., FITZPATRICK, L., RUGGIERO, F. & KOLTUN, W. A. 2007. Loss of the tight junction protein ZO-1 in dextran sulfate sodium induced colitis. *J Surg Res,* 140**,** 12-9.

PRIMIANO, M. J., LEFKER, B. A., BOWMAN, M. R., BREE, A. G., HUBEAU, C., BONIN, P. D., MANGAN, M., DOWER, K., MONKS, B. G., CUSHING, L., WANG, S., GUZOVA, J., JIAO, A., LIN, L. L., LATZ, E., HEPWORTH, D. & HALL, J. P. 2016. Efficacy and Pharmacology of the NLRP3 Inflammasome Inhibitor CP-456,773 (CRID3) in Murine Models of Dermal and Pulmonary Inflammation. *J Immunol,* 197**,** 2421-33.

PULLAN, R. D., THOMAS, G. A., RHODES, M., NEWCOMBE, R. G., WILLIAMS, G. T., ALLEN, A. & RHODES, J. 1994. Thickness of adherent mucus gel on colonic mucosa in humans and its relevance to colitis. *Gut,* 35**,** 353-9.

RAAB, Y., HALLGREN, R., KNUTSON, L., KROG, M. & GERDIN, B. 1992. A technique for segmental rectal and colonic perfusion in humans. *Am J Gastroenterol,* 87**,** 1453-9.

RAHMAN, A. A., ROBINSON, A. M., BROOKES, S. J. H., ERI, R. & NURGALI, K. 2016. Rectal prolapse in Winnie mice with spontaneous chronic colitis: changes in intrinsic and extrinsic innervation of the rectum. *Cell Tissue Res,* 366**,** 285-299.

RAHMAN, A. A., ROBINSON, A. M., JOVANOVSKA, V., ERI, R. & NURGALI, K. 2015a. Alterations in the distal colon innervation in Winnie mouse model of spontaneous chronic colitis. *Cell and Tissue Research,* 362**,** 497-512.

RAHMAN, A. A., ROBINSON, A. M., JOVANOVSKA, V., ERI, R. & NURGALI, K. 2015b. Alterations in the distal colon innervation in Winnie mouse model of spontaneous chronic colitis. *Cell Tissue Res,* 362**,** 497-512.

RAJAMAKI, K., LAPPALAINEN, J., OORNI, K., VALIMAKI, E., MATIKAINEN, S., KOVANEN, P. T. & EKLUND, K. K. 2010. Cholesterol crystals activate the NLRP3 inflammasome in human macrophages: a novel link between cholesterol metabolism and inflammation. *PLoS One,* 5**,** e11765.

RANDALL-DEMLLO, S., FERNANDO, R., BRAIN, T., SOHAL, S. S., COOK, A. L., GUVEN, N., KUNDE, D., SPRING, K. & ERI, R. 2016. Characterisation of colonic dysplasia-like epithelial atypia in murine colitis. *World Journal of Gastroenterology,* 22**,** 8334-8348.

RANSON, N. & ERI, R. 2013. The Role of Inflammasomes in Intestinal Inflammation. *American Journal of Medical and Biological Research,* 1**,** 64-76.

RANSON, N., KUNDE, D. & ERI, R. 2017. Regulation and Sensing of Inflammasomes and Their Impact on Intestinal Health. *Int J Mol Sci,* 18.

RATHINAM, V. A., VANAJA, S. K. & FITZGERALD, K. A. 2012a. Regulation of inflammasome signaling. *Nat Immunol,* 13**,** 333-42.

RATHINAM, V. A., VANAJA, S. K., WAGGONER, L., SOKOLOVSKA, A., BECKER, C., STUART, L. M., LEONG, J. M. & FITZGERALD, K. A. 2012b. TRIF licenses caspase-11-dependent NLRP3 inflammasome activation by gram-negative bacteria. *Cell,* 150**,** 606-19.

REDDY, B. S., EL-BAYOUMY, K. & LOUIS, Y. M. 1985. Germfree animal as a tool to study role of gut microflora and nutrition in cancer. *Prog Clin Biol Res,* 181**,** 293-6.

REQUENA, T., MARTINEZ-CUESTA, M. C. & PELAEZ, C. 2018. Diet and microbiota linked in health and disease. *Food Funct,* 9**,** 688-704.

REUTER, B. K. & PIZARRO, T. T. 2004. Commentary: the role of the IL-18 system and other members of the IL-1R/TLR superfamily in innate mucosal immunity and the pathogenesis of inflammatory bowel disease: friend or foe? *Eur J Immunol,* 34**,** 2347-55.

RICHARD, M. L., LIGUORI, G., LAMAS, B., BRANDI, G., DA COSTA, G., HOFFMANN, T. W., PIERLUIGI DI SIMONE, M., CALABRESE, C., POGGIOLI, G., LANGELLA, P., CAMPIERI, M. & SOKOL, H. 2018. Mucosa-associated microbiota dysbiosis in colitis associated cancer. *Gut Microbes,* 9**,** 131-142.

RISQUES, R. A., LAI, L. A., BRENTNALL, T. A., LI, L., FENG, Z., GALLAHER, J., MANDELSON, M. T., POTTER, J. D., BRONNER, M. P. & RABINOVITCH, P. S. 2008. Ulcerative colitis is a disease of accelerated colon aging: evidence from telomere attrition and DNA damage. *Gastroenterology,* 135**,** 410-8.

ROBINSON, A. M., GONDALIA, S. V., KARPE, A. V., ERI, R., BEALE, D. J., MORRISON, P. D., PALOMBO, E. A. & NURGALI, K. 2016a. Fecal Microbiota and Metabolome in a Mouse Model of Spontaneous Chronic Colitis: Relevance to Human Inflammatory Bowel Disease. *Inflammatory Bowel Diseases,* 22**,** 2767-2787.

ROBINSON, A. M., RAHMAN, A. A., CARBONE, S. E., RANDALL-DEMLLO, S., FILIPPONE, R., BORNSTEIN, J. C., ERI, R. & NURGALI, K. 2016b. Alterations of colonic function in the Winnie mouse model of spontaneous chronic colitis. *American Journal of Physiology - Gastrointestinal and Liver Physiology*.

ROBINSON, A. M., RAHMAN, A. A., CARBONE, S. E., RANDALL-DEMLLO, S., FILIPPONE, R., BORNSTEIN, J. C., ERI, R. & NURGALI, K. 2017. Alterations of colonic function in the Winnie mouse model of spontaneous chronic colitis. *American Journal of Physiology-Gastrointestinal and Liver Physiology,* 312**,** G85-G102.

RODRIGUEZ-SALAS, N., DOMINGUEZ, G., BARDERAS, R., MENDIOLA, M., GARCIA-ALBENIZ, X., MAUREL, J. & BATLLE, J. F. 2017. Clinical relevance of colorectal cancer molecular subtypes. *Crit Rev Oncol Hematol,* 109**,** 9-19.

ROESSNER, A., KUESTER, D., MALFERTHEINER, P. & SCHNEIDER-STOCK, R. 2008. Oxidative stress in ulcerative colitis-associated carcinogenesis. *Pathol Res Pract,* 204**,** 511-24.

ROGAKOU, E. P., PILCH, D. R., ORR, A. H., IVANOVA, V. S. & BONNER, W. M. 1998. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem,* 273**,** 5858-68.

RONCUCCI, L., MORA, E., MARIANI, F., BURSI, S., PEZZI, A., ROSSI, G., PEDRONI, M., LUPPI, D., SANTORO, L., MONNI, S., MANENTI, A., BERTANI, A., MERIGHI, A., BENATTI, P., DI GREGORIO, C. & DE LEON, P. M. 2008. Myeloperoxidase-positive cell infiltration in colorectal carcinogenesis as indicator of colorectal cancer risk. *Cancer Epidemiol Biomarkers Prev,* 17**,** 2291-7.

ROUND, J. L. & MAZMANIAN, S. K. 2009. The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol,* 9**,** 313-23.

ROXBURGH, C. S., SALMOND, J. M., HORGAN, P. G., OIEN, K. A. & MCMILLAN, D. C. 2009. Tumour inflammatory infiltrate predicts survival following curative resection for node-negative colorectal cancer. *Eur J Cancer,* 45**,** 2138-45.

RUBIN, D. C., SHAKER, A. & LEVIN, M. S. 2012. Chronic intestinal inflammation: inflammatory bowel disease and colitis-associated colon cancer. *Front Immunol,* 3**,** 107.

RUTTER, M. D., SAUNDERS, B. P., WILKINSON, K. H., RUMBLES, S., SCHOFIELD, G., KAMM, M. A., WILLIAMS, C. B., PRICE, A. B., TALBOT, I. C. & FORBES, A. 2004. Cancer surveillance in longstanding ulcerative colitis: endoscopic appearances help predict cancer risk. *Gut,* 53**,** 1813-6.

SALARO, E., RAMBALDI, A., FALZONI, S., AMOROSO, F. S., FRANCESCHINI, A., SARTI, A. C., BONORA, M., CAVAZZINI, F., RIGOLIN, G. M., CICCONE, M., AUDRITO, V., DEAGLIO, S., PELEGRIN, P., PINTON, P., CUNEO, A. & DI VIRGILIO, F. 2016. Involvement of the P2X7-NLRP3 axis in leukemic cell proliferation and death. *Scientific Reports,* 6**,** 13.

SALCEDO, R., WORSCHECH, A., CARDONE, M., JONES, Y., GYULAI, Z., DAI, R. M., WANG, E., MA, W., HAINES, D., O'HUIGIN, C., MARINCOLA, F. M. & TRINCHIERI, G. 2010. MyD88-mediated signaling prevents development of adenocarcinomas of the colon: role of interleukin 18. *J Exp Med,* 207**,** 1625-36.

SANAPAREDDY, N., LEGGE, R. M., JOVOV, B., MCCOY, A., BURCAL, L., ARAUJO-PEREZ, F., RANDALL, T. A., GALANKO, J., BENSON, A., SANDLER, R. S., RAWLS, J. F., ABDO, Z., FODOR, A. A. & KEKU, T. O. 2012. Increased rectal microbial richness is associated with the presence of colorectal adenomas in humans. *Isme j,* 6**,** 1858-68.

SÁNCHEZ-FIDALGO, S., VILLEGAS, I., CÁRDENO, A., TALERO, E., SÁNCHEZ-HIDALGO, M., MOTILVA, V. & ALARCÓN DE LA LASTRA, C. 2010. Extra-virgin olive oil-enriched diet modulates DSS-colitis-associated colon carcinogenesis in mice. *Clinical Nutrition,* 29**,** 663-673.

SANSONE, S. A., FAN, T., GOODACRE, R., GRIFFIN, J. L., HARDY, N. W., KADDURAH-DAOUK, R., KRISTAL, B. S., LINDON, J., MENDES, P., MORRISON, N., NIKOLAU, B., ROBERTSON, D., SUMNER, L. W., TAYLOR, C., VAN DER WERF, M., VAN OMMEN, B. & FIEHN, O. 2007. The metabolomics standards initiative. *Nat Biotechnol,* 25**,** 846-8.

SARTOR, R. B. 1994. Cytokines in intestinal inflammation: pathophysiological and clinical considerations. *Gastroenterology,* 106**,** 533-9.

SAWA, T. & OHSHIMA, H. 2006. Nitrative DNA damage in inflammation and its possible role in carcinogenesis. *Nitric Oxide,* 14**,** 91-100.

SCHILLING, J. D., MACHKOVECH, H. M., HE, L., SIDHU, R., FUJIWARA, H., WEBER, K., ORY, D. S. & SCHAFFER, J. E. 2013. Palmitate and lipopolysaccharide trigger synergistic ceramide production in primary macrophages. *J Biol Chem,* 288**,** 2923-32.

SCHOULTZ, I., VERMA, D., HALFVARSSON, J., TORKVIST, L., FREDRIKSON, M., SJOQVIST, U., LORDAL, M., TYSK, C., LERM, M., SODERKVIST, P. & SODERHOLM, J. D. 2009. Combined polymorphisms in genes encoding the inflammasome components NALP3 and CARD8 confer susceptibility to Crohn's disease in Swedish men. *Am J Gastroenterol,* 104**,** 1180-8.

SCHRODER, K. & TSCHOPP, J. 2010. The inflammasomes. *Cell,* 140**,** 821-32.

SEDELNIKOVA, O. A. & BONNER, W. M. 2006. GammaH2AX in cancer cells: a potential biomarker for cancer diagnostics, prediction and recurrence. *Cell Cycle,* 5**,** 2909-13.

SEO, S. U., KAMADA, N., MUNOZ-PLANILLO, R., KIM, Y. G., KIM, D., KOIZUMI, Y., HASEGAWA, M., HIMPSL, S. D., BROWNE, H. P., LAWLEY, T. D., MOBLEY, H. L., INOHARA, N. & NUNEZ, G. 2015. Distinct Commensals Induce Interleukin-1beta via NLRP3 Inflammasome in Inflammatory Monocytes to Promote Intestinal Inflammation in Response to Injury. *Immunity,* 42**,** 744-55.

SEREGIN, S. S., GOLOVCHENKO, N., SCHAF, B., CHEN, J., PUDLO, N. A., MITCHELL, J., BAXTER, N. T., ZHAO, L., SCHLOSS, P. D., MARTENS, E. C., EATON, K. A. & CHEN, G. Y. 2017. NLRP6 Protects Il10(-/-) Mice from Colitis by Limiting Colonization of Akkermansia muciniphila. *Cell Rep,* 19**,** 733-745.

SHAKED, H., HOFSETH, L. J., CHUMANEVICH, A., CHUMANEVICH, A. A., WANG, J., WANG, Y., TANIGUCHI, K., GUMA, M., SHENOUDA, S., CLEVERS, H., HARRIS, C. C. & KARIN, M. 2012. Chronic epithelial NF-kappaB activation accelerates APC loss and intestinal tumor initiation through iNOS up-regulation. *Proc Natl Acad Sci U S A,* 109**,** 14007-12.

SHARKEY, K. A. & KROESE, A. B. A. 2001. Consequences of intestinal inflammation on the enteric nervous system: Neuronal activation induced by inflammatory mediators. *The Anatomical Record,* 262**,** 79-90.

SHENG, H., SHAO, J., TOWNSEND, C. M., JR. & EVERS, B. M. 2003. Phosphatidylinositol 3-kinase mediates proliferative signals in intestinal epithelial cells. *Gut,* 52**,** 1472-8.

SHI, C. Y., FAN, Y., LIU, B. & LOU, W. H. 2013. HIF1 contributes to hypoxia-induced pancreatic cancer cells invasion via promoting QSOX1 expression. *Cell Physiol Biochem,* 32**,** 561-8.

SHI, H. X., WANG, Y., LI, X. H., ZHAN, X. M., TANG, M., FINA, M., SU, L. J., PRATT, D., BU, C. H., HILDEBRAND, S., LYON, S., SCOTT, L., QUAN, J. X., SUN, Q. H., RUSSELL, J., ARNETT, S., JUREK, P., CHEN, D., KRAVCHENKO, V. V., MATHISON, J. C., MORESCO, E. M. Y., MONSON,

N. L., ULEVITCH, R. J. & BEUTLER, B. 2016. NLRP3 activation and mitosis are mutually exclusive events coordinated by NEK7, a new inflammasome component. *Nature Immunology,* 17**,** 250-258.

SHIMADA, K., CROTHER, T. R., KARLIN, J., DAGVADORJ, J., CHIBA, N., CHEN, S., RAMANUJAN, V. K., WOLF, A. J., VERGNES, L., OJCIUS, D. M., RENTSENDORJ, A., VARGAS, M., GUERRERO, C., WANG, Y., FITZGERALD, K. A., UNDERHILL, D. M., TOWN, T. & ARDITI, M. 2012. Oxidized mitochondrial DNA activates the NLRP3 inflammasome during apoptosis. *Immunity,* 36**,** 401-14.

SHTUTMAN, M., ZHURINSKY, J., SIMCHA, I., ALBANESE, C., D'AMICO, M., PESTELL, R. & BEN-ZE'EV, A. 1999. The cyclin D1 gene is a target of the β-catenin/LEF-1 pathway. *Proceedings of the National Academy of Sciences,* 96**,** 5522.

SIEGEL, R., DESANTIS, C. & JEMAL, A. 2014. Colorectal cancer statistics, 2014. *CA Cancer J Clin,* 64**,** 104-17.

SIEGEL, R. L., MILLER, K. D. & JEMAL, A. 2016. Cancer statistics, 2016. *CA Cancer J Clin,* 66**,** 7-30.

SIEGMUND, B. 2010a. Interleukin-18 in Intestinal Inflammation: Friend and Foe? *Immunity,* 32**,** 300-302.

SIEGMUND, B. 2010b. Interleukin-18 in intestinal inflammation: friend and foe? *Immunity,* 32**,** 300-2.

SIEGMUND, B., FANTUZZI, G., RIEDER, F., GAMBONI-ROBERTSON, F., LEHR, H. A., HARTMANN, G., DINARELLO, C. A., ENDRES, S. & EIGLER, A. 2001a. Neutralization of interleukin-18 reduces severity in murine colitis and intestinal IFN-gamma and TNF-alpha production. *Am J Physiol Regul Integr Comp Physiol,* 281**,** R1264-73.

SIEGMUND, B., LEHR, H.-A., FANTUZZI, G. & DINARELLO, C. A. 2001b. IL-1β-converting enzyme (caspase-1) in intestinal inflammation. *Proceedings of the National Academy of Sciences,* 98**,** 13249-13254.

SINGH, N., GURAV, A., SIVAPRAKASAM, S., BRADY, E., PADIA, R., SHI, H., THANGARAJU, M., PRASAD, P. D., MANICASSAMY, S., MUNN, D. H., LEE, J. R., OFFERMANNS, S. & GANAPATHY, V. 2014. Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. *Immunity,* 40**,** 128-39.

SIVAKUMAR, P. V., WESTRICH, G. M., KANALY, S., GARKA, K., BORN, T. L., DERRY, J. M. & VINEY, J. L. 2002. Interleukin 18 is a primary mediator of the inflammation associated with dextran sulphate sodium induced colitis: blocking interleukin 18 attenuates intestinal damage. *Gut,* 50**,** 812-20.

SMITH, P. M., HOWITT, M. R., PANIKOV, N., MICHAUD, M., GALLINI, C. A., BOHLOOLY-Y, M., GLICKMAN, J. N. & GARRETT, W. S. 2013. The Microbial Metabolites, Short-Chain Fatty Acids, Regulate Colonic T_{reg} Cell Homeostasis. *Science,* 341**,** 569.

SNOW, B. J., ROLFE, F. L., LOCKHART, M. M., FRAMPTON, C. M., O'SULLIVAN, J. D., FUNG, V., SMITH, R. A., MURPHY, M. P. & TAYLOR, K. M. 2010. A double-blind, placebo-controlled study to assess the mitochondria-targeted antioxidant MitoQ as a disease-modifying therapy in Parkinson's disease. *Mov Disord,* 25**,** 1670-4.

SOHN, J. J., SCHETTER, A. J., YFANTIS, H. G., RIDNOUR, L. A., HORIKAWA, I., KHAN, M. A., ROBLES, A. I., HUSSAIN, S. P., GOTO, A., BOWMAN, E. D., HOFSETH, L. J., BARTKOVA, J., BARTEK, J., WOGAN, G. N., WINK, D. A. & HARRIS, C. C. 2012. Macrophages, nitric oxide and

microRNAs are associated with DNA damage response pathway and senescence in inflammatory bowel disease. *PLoS One,* 7**,** e44156.

SOLEM, C. A., LOFTUS, E. V., JR., TREMAINE, W. J., HARMSEN, W. S., ZINSMEISTER, A. R. & SANDBORN, W. J. 2005. Correlation of C-reactive protein with clinical, endoscopic, histologic, and radiographic activity in inflammatory bowel disease. *Inflamm Bowel Dis,* 11**,** 707-12.

SONG-ZHAO, G. X., SRINIVASAN, N., POTT, J., BABAN, D., FRANKEL, G. & MALOY, K. J. 2013. Nlrp3 activation in the intestinal epithelium protects against a mucosal pathogen. *Mucosal Immunology,* 7**,** 763.

STAMBOLIC, V., SUZUKI, A., DE LA POMPA, J. L., BROTHERS, G. M., MIRTSOS, C., SASAKI, T., RULAND, J., PENNINGER, J. M., SIDEROVSKI, D. P. & MAK, T. W. 1998. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell,* 95**,** 29-39.

STORSTEEN, K. A., KERNOHAN, J. W. & BARGEN, J. A. 1953. The myenteric plexus in chronic ulcerative colitis. *Surg Gynecol Obstet,* 97**,** 335-43.

STRASSER, A., CORY, S. & ADAMS, J. M. 2011. Deciphering the rules of programmed cell death to improve therapy of cancer and other diseases. *Embo j,* 30**,** 3667-83.

STROBER, W. 2013. Impact of the gut microbiome on mucosal inflammation. *Trends in Immunology,* 34**,** 423–430.

STURLAN, S., OBERHUBER, G., BEINHAUER, B. G., TICHY, B., KAPPEL, S., WANG, J. & ROGY, M. A. 2001. Interleukin-10-deficient mice and inflammatory bowel disease associated cancer development. *Carcinogenesis,* 22**,** 665-71.

STUYT, R. J., NETEA, M. G., GEIJTENBEEK, T. B., KULLBERG, B. J., DINARELLO, C. A. & VAN DER MEER, J. W. 2003. Selective regulation of intercellular adhesion molecule-1 expression by interleukin-18 and interleukin-12 on human monocytes. *Immunology,* 110**,** 329-34.

SUN, H., ZHANG, A., YAN, G., PIAO, C., LI, W., SUN, C., WU, X., LI, X., CHEN, Y. & WANG, X. 2013. Metabolomic analysis of key regulatory metabolites in hepatitis C virus-infected tree shrews. *Mol Cell Proteomics,* 12**,** 710-9.

SUN, Y., ZHAO, Y., YAO, J., ZHAO, L., WU, Z., WANG, Y., PAN, D., MIAO, H., GUO, Q. & LU, N. 2015. Wogonoside protects against dextran sulfate sodium-induced experimental colitis in mice by inhibiting NF-kappaB and NLRP3 inflammasome activation. *Biochem Pharmacol,* 94**,** 142-54.

SUTTERWALA, F. S., OGURA, Y., SZCZEPANIK, M., LARA-TEJERO, M., LICHTENBERGER, G. S., GRANT, E. P., BERTIN, J., COYLE, A. J., GALAN, J. E., ASKENASE, P. W. & FLAVELL, R. A. 2006. Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1. *Immunity,* 24**,** 317-27.

SUZUKI, T., MOTOHASHI, H. & YAMAMOTO, M. 2013. Toward clinical application of the Keap1-Nrf2 pathway. *Trends Pharmacol Sci,* 34**,** 340-6.

SZTEIN, J. M., KASTENMAYER, R. J. & PERDUE, K. A. 2011. Pathogen-Free Mouse Rederivation by IVF, Natural Mating and Hysterectomy. *In:* PEASE, S. & SAUNDERS, T. L. (eds.) *Advanced Protocols for Animal Transgenesis: An ISTT Manual.* Berlin, Heidelberg: Springer Berlin Heidelberg.

TAKAGI, H., KANAI, T., OKAZAWA, A., KISHI, Y., SATO, T., TAKAISHI, H., INOUE, N., OGATA, H., IWAO, Y., HOSHINO, K., TAKEDA, K., AKIRA, S., WATANABE, M., ISHII, H. & HIBI, T. 2003.

Contrasting action of IL-12 and IL-18 in the development of dextran sodium sulphate colitis in mice. *Scand J Gastroenterol,* 38**,** 837-44.

TAKEDA, K., TSUTSUI, H., YOSHIMOTO, T., ADACHI, O., YOSHIDA, N., KISHIMOTO, T., OKAMURA, H., NAKANISHI, K. & AKIRA, S. 1998. Defective NK cell activity and Th1 response in IL-18-deficient mice. *Immunity,* 8**,** 383-90.

TALEBAN, S., COLOMBEL, J.-F., MOHLER, M. J. & FAIN, M. J. 2015. Inflammatory Bowel Disease and the Elderly: A Review. *Journal of Crohn's and Colitis,* 9**,** 507-515.

TAMURA, K., FUKUDA, Y., SASHIO, H., TAKEDA, N., BAMBA, H., KOSAKA, T., FUKUI, S., SAWADA, K., TAMURA, K., SATOMI, M., YAMADA, T., YAMAMURA, T., YAMAMOTO, Y., FURUYAMA, J., OKAMURA, H. & SHIMOYAMA, T. 2002. IL18 polymorphism is associated with an increased risk of Crohn's disease. *J Gastroenterol,* 37 Suppl 14**,** 111-6.

TANAKA, T., KOHNO, H., SUZUKI, R., YAMADA, Y., SUGIE, S. & MORI, H. 2003. A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. *Cancer Sci,* 94**,** 965-73.

TATE, M. D., ONG, J. D. H., DOWLING, J. K., MCAULEY, J. L., ROBERTSON, A. B., LATZ, E., DRUMMOND, G. R., COOPER, M. A., HERTZOG, P. J. & MANSELL, A. 2016. Reassessing the role of the NLRP3 inflammasome during pathogenic influenza A virus infection via temporal inhibition. *Scientific Reports,* 6.

TEN HOVE, T., CORBAZ, A., AMITAI, H., ALONI, S., BELZER, I., GRABER, P., DRILLENBURG, P., VAN DEVENTER, S. J., CHVATCHKO, Y. & TE VELDE, A. A. 2001. Blockade of endogenous IL-18 ameliorates TNBS-induced colitis by decreasing local TNF-alpha production in mice. *Gastroenterology,* 121**,** 1372-9.

TERZIĆ, J., GRIVENNIKOV, S., KARIN, E. & KARIN, M. 2010. Inflammation and Colon Cancer. *Gastroenterology,* 138**,** 2101-2114.e5.

THOMAS, T. K., WILL, P. C., SRIVASTAVA, A., WILSON, C. L., HARBISON, M., LITTLE, J., CHESONIS, R. S., PIGNATELLO, M., SCHMOLZE, D., SYMINGTON, J., KILIAN, P. L. & THOMPSON, R. C. 1991. Evaluation of an interleukin-1 receptor antagonist in the rat acetic acid-induced colitis model. *Agents and Actions,* 34**,** 187-190.

TIAN, Z., LIU, Y., YANG, B., ZHANG, J., HE, H., GE, H., WU, Y. & SHEN, Z. 2016. Astagalus Polysaccharide Attenuates Murine Colitis through Inhibiton of the NLRP3 Inflammasome. *Planta Med*.

TJALSMA, H., BOLEIJ, A., MARCHESI, J. R. & DUTILH, B. E. 2012. A bacterial driver-passenger model for colorectal cancer: beyond the usual suspects. *Nat Rev Microbiol,* 10**,** 575-82.

TRIANTAFILLIDIS, J. K., NASIOULAS, G. & KOSMIDIS, P. A. 2009. Colorectal Cancer and Inflammatory Bowel Disease: Epidemiology, Risk Factors, Mechanisms of Carcinogenesis and Prevention Strategies. *Anticancer Research,* 29**,** 2727-2737.

TRUTMANN, M., TERRACCIANO, L., NOPPEN, C., KLOTH, J., KASPAR, M., PETERLI, R., TONDELLI, P., SCHAEFFER, C., ZAJAC, P., HEBERER, M. & SPAGNOLI, G. C. 1998. GM-CSF gene expression and protein production in human colorectal cancer cell lines and clinical tumor specimens. *Int J Cancer,* 77**,** 378-85.

UMAR, A., BOISSEAU, M., YUSUP, A., UPUR, H., BEGAUD, B. & MOORE, N. 2004. Interactions between aspirin and COX-2 inhibitors or NSAIDs in a rat thrombosis model. *Fundam Clin Pharmacol,* 18**,** 559-63.

UNGERBÄCK, J., BELENKI, D., JAWAD UL-HASSAN, A., FREDRIKSON, M., FRANSÉN, K., ELANDER, N., VERMA, D. & SÖDERKVIST, P. 2012. Genetic variation and alterations of genes involved in NFκB/TNFAIP3- and NLRP3-inflammasome signaling affect susceptibility and outcome of colorectal cancer. *Carcinogenesis,* 33**,** 2126-2134.

URDINGUIO, R. G., FERNANDEZ, A. F., MONCADA-PAZOS, A., HUIDOBRO, C., RODRIGUEZ, R. M., FERRERO, C., MARTINEZ-CAMBLOR, P., OBAYA, A. J., BERNAL, T., PARRA-BLANCO, A., RODRIGO, L., SANTACANA, M., MATIAS-GUIU, X., SOLDEVILLA, B., DOMINGUEZ, G., BONILLA, F., CAL, S., LOPEZ-OTIN, C. & FRAGA, M. F. 2013. Immune-dependent and independent antitumor activity of GM-CSF aberrantly expressed by mouse and human colorectal tumors. *Cancer Res,* 73**,** 395-405.

VAN DE VEERDONK, F. L., NETEA, M. G., DINARELLO, C. A. & JOOSTEN, L. A. 2011. Inflammasome activation and IL-1beta and IL-18 processing during infection. *Trends Immunol,* 32**,** 110-6.

VAN DER FLIER, L. G., SABATES-BELLVER, J., OVING, I., HAEGEBARTH, A., DE PALO, M., ANTI, M., VAN GIJN, M. E., SUIJKERBUIJK, S., VAN DE WETERING, M., MARRA, G. & CLEVERS, H. 2007. The Intestinal Wnt/TCF Signature. *Gastroenterology,* 132**,** 628-32.

VAN DER SLUIS, M., DE KONING, B. A., DE BRUIJN, A. C., VELCICH, A., MEIJERINK, J. P., VAN GOUDOEVER, J. B., BULLER, H. A., DEKKER, J., VAN SEUNINGEN, I., RENES, I. B. & EINERHAND, A. W. 2006. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. *Gastroenterology,* 131**,** 117-29.

VAN HAUWERMEIREN, F. & LAMKANFI, M. 2016. The NEK-sus of the NLRP3 inflammasome. *Nature Immunology,* 17**,** 223-224.

VAN HOUT, G. P., BOSCH, L., ELLENBROEK, G. H., DE HAAN, J. J., VAN SOLINGE, W. W., COOPER, M. A., ARSLAN, F., DE JAGER, S. C., ROBERTSON, A. A., PASTERKAMP, G. & HOEFER, I. E. 2016. The selective NLRP3-inflammasome inhibitor MCC950 reduces infarct size and preserves cardiac function in a pig model of myocardial infarction. *Eur Heart J*.

VANDANMAGSAR, B., YOUM, Y. H., RAVUSSIN, A., GALGANI, J. E., STADLER, K., MYNATT, R. L., RAVUSSIN, E., STEPHENS, J. M. & DIXIT, V. D. 2011. The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. *Nat Med,* 17**,** 179-88.

VELA, J. M., MOLINA-HOLGADO, E., AREVALO-MARTIN, A., ALMAZAN, G. & GUAZA, C. 2002. Interleukin-1 regulates proliferation and differentiation of oligodendrocyte progenitor cells. *Mol Cell Neurosci,* 20**,** 489-502.

VELCICH, A., YANG, W., HEYER, J., FRAGALE, A., NICHOLAS, C., VIANI, S., KUCHERLAPATI, R., LIPKIN, M., YANG, K. & AUGENLICHT, L. 2002. Colorectal Cancer in Mice Genetically Deficient in the Mucin Muc2. *Science,* 295**,** 1726.

VEMURI, R., SHINDE, T., GUNDAMARAJU, R., GONDALIA, S., KARPE, A., BEALE, D., MARTONI, C. & ERI, R. 2018. Lactobacillus acidophilus DDS-1 Modulates the Gut Microbiota and Improves Metabolic Profiles in Aging Mice. *Nutrients,* 10**,** 1255.

VIALA, J., CHAPUT, C., BONECA, I. G., CARDONA, A., GIRARDIN, S. E., MORAN, A. P., ATHMAN, R., MEMET, S., HUERRE, M. R., COYLE, A. J., DISTEFANO, P. S., SANSONETTI, P. J., LABIGNE, A., BERTIN, J., PHILPOTT, D. J. & FERRERO, R. L. 2004. Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. *Nat Immunol,* 5**,** 1166-74.

VILLANI, A. C., LEMIRE, M., FORTIN, G., LOUIS, E., SILVERBERG, M. S., COLLETTE, C., BABA, N., LIBIOULLE, C., BELAICHE, J., BITTON, A., GAUDET, D., COHEN, A., LANGELIER, D., FORTIN, P. R., WITHER, J. E., SARFATI, M., RUTGEERTS, P., RIOUX, J. D., VERMEIRE, S., HUDSON, T.

J. & FRANCHIMONT, D. 2009. Common variants in the NLRP3 region contribute to Crohn's disease susceptibility. *Nat Genet,* 41**,** 71-6.

VLADIMER, G. I., WENG, D., PAQUETTE, S. W., VANAJA, S. K., RATHINAM, V. A., AUNE, M. H., CONLON, J. E., BURBAGE, J. J., PROULX, M. K., LIU, Q., REED, G., MECSAS, J. C., IWAKURA, Y., BERTIN, J., GOGUEN, J. D., FITZGERALD, K. A. & LIEN, E. 2012. The NLRP12 inflammasome recognizes Yersinia pestis. *Immunity,* 37**,** 96-107.

VORONOV, E., DOTAN, S., KRELIN, Y., SONG, X., ELKABETS, M., CARMI, Y., RIDER, P., IDAN, C., ROMZOVA, M., KAPLANOV, I. & APTE, R. N. 2013. Unique Versus Redundant Functions of IL-1α and IL-1β in the Tumor Microenvironment. *Frontiers in immunology,* 4**,** 177-177.

VORONOV, E., SHOUVAL, D. S., KRELIN, Y., CAGNANO, E., BENHARROCH, D., IWAKURA, Y., DINARELLO, C. A. & APTE, R. N. 2003. IL-1 is required for tumor invasiveness and angiogenesis. *Proceedings of the National Academy of Sciences,* 100**,** 2645.

WALDNER, M. J. & NEURATH, M. F. 2015. Mechanisms of Immune Signaling in Colitis-Associated Cancer. *Cell Mol Gastroenterol Hepatol,* 1**,** 6-16.

WALDNER, M. J., WIRTZ, S., JEFREMOW, A., WARNTJEN, M., NEUFERT, C., ATREYA, R., BECKER, C., WEIGMANN, B., VIETH, M., ROSE-JOHN, S. & NEURATH, M. F. 2010a. VEGF receptor signaling links inflammation and tumorigenesis in colitis-associated cancer. *The Journal of experimental medicine,* 207**,** 2855-2868.

WALDNER, M. J., WIRTZ, S., JEFREMOW, A., WARNTJEN, M., NEUFERT, C., ATREYA, R., BECKER, C., WEIGMANN, B., VIETH, M., ROSE-JOHN, S. & NEURATH, M. F. 2010b. VEGF receptor signaling links inflammation and tumorigenesis in colitis-associated cancer. *J Exp Med,* 207**,** 2855-68.

WANG, K., KIM, M. K., DI CARO, G., WONG, J., SHALAPOUR, S., WAN, J., ZHANG, W., ZHONG, Z., SANCHEZ-LOPEZ, E., WU, L. W., TANIGUCHI, K., FENG, Y., FEARON, E., GRIVENNIKOV, S. I. & KARIN, M. 2014a. Interleukin-17 receptor a signaling in transformed enterocytes promotes early colorectal tumorigenesis. *Immunity,* 41**,** 1052-63.

WANG, L., WANG, Y., SONG, Z., CHU, J. & QU, X. 2015a. Deficiency of interferon-gamma or its receptor promotes colorectal cancer development. *J Interferon Cytokine Res,* 35**,** 273-80.

WANG, Q. S., PAPANIKOLAOU, A., SABOURIN, C. L. & ROSENBERG, D. W. 1998. Altered expression of cyclin D1 and cyclin-dependent kinase 4 in azoxymethane-induced mouse colon tumorigenesis. *Carcinogenesis,* 19**,** 2001-6.

WANG, R., HASNAIN, S. Z., TONG, H., DAS, I., CHEN, A. C. H., OANCEA, I., PROCTOR, M., FLORIN, T. H., ERI, R. D. & MCGUCKIN, M. A. 2015b. Neutralizing IL-23 Is Superior to Blocking IL-17 in Suppressing Intestinal Inflammation in a Spontaneous Murine Colitis Model. *Inflammatory Bowel Diseases,* 21**,** 973-984.

WANG, S., LIU, Z., WANG, L. & ZHANG, X. 2009. NF-kappaB signaling pathway, inflammation and colorectal cancer. *Cell Mol Immunol,* 6**,** 327-34.

WANG, T., CAI, G., QIU, Y., FEI, N., ZHANG, M., PANG, X., JIA, W., CAI, S. & ZHAO, L. 2012. Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers. *Isme j,* 6**,** 320-9.

WANG, Y., HAN, G., WANG, K., LIU, G., WANG, R., XIAO, H., LI, X., HOU, C., SHEN, B., GUO, R., LI, Y. & CHEN, G. 2014b. Tumor-derived GM-CSF promotes inflammatory colon carcinogenesis via stimulating epithelial release of VEGF. *Cancer Res,* 74**,** 716-26.

WANG, Y., VAN BOXEL-DEZAIRE, A. H., CHEON, H., YANG, J. & STARK, G. R. 2013. STAT3 activation in response to IL-6 is prolonged by the binding of IL-6 receptor to EGF receptor. *Proc Natl Acad Sci U S A,* 110**,** 16975-80.

WANG, Y., WANG, K., HAN, G. C., WANG, R. X., XIAO, H., HOU, C. M., GUO, R. F., DOU, Y., SHEN, B. F., LI, Y. & CHEN, G. J. 2014c. Neutrophil infiltration favors colitis-associated tumorigenesis by activating the interleukin-1 (IL-1)/IL-6 axis. *Mucosal Immunol,* 7**,** 1106-15.

WILLIAMS, N. S., GAYNOR, R. B., SCOGGIN, S., VERMA, U., GOKASLAN, T., SIMMANG, C., FLEMING, J., TAVANA, D., FRENKEL, E. & BECERRA, C. 2003. Identification and validation of genes involved in the pathogenesis of colorectal cancer using cDNA microarrays and RNA interference. *Clin Cancer Res,* 9**,** 931-46.

WILLIAMS, T. M., LEETH, R. A., ROTHSCHILD, D. E., MCDANIEL, D. K., COUTERMARSH-OTT, S. L., SIMMONS, A. E., KABLE, K. H., HEID, B. & ALLEN, I. C. 2015. Caspase-11 attenuates gastrointestinal inflammation and experimental colitis pathogenesis. *Am J Physiol Gastrointest Liver Physiol,* 308**,** G139-50.

WILSON, J., HAIR, C., KNIGHT, R., CATTO-SMITH, A., BELL, S., KAMM, M., DESMOND, P., MCNEIL, J. & CONNELL, W. 2010. High incidence of inflammatory bowel disease in Australia: a prospective population-based Australian incidence study. *Inflamm Bowel Dis,* 16**,** 1550-6.

WILSON, J. E., PETRUCELLI, A. S., CHEN, L., KOBLANSKY, A. A., TRUAX, A. D., OYAMA, Y., ROGERS, A. B., BRICKEY, W. J., WANG, Y., SCHNEIDER, M., MUHLBAUER, M., CHOU, W. C., BARKER, B. R., JOBIN, C., ALLBRITTON, N. L., RAMSDEN, D. A., DAVIS, B. K. & TING, J. P. 2015. Inflammasome-independent role of AIM2 in suppressing colon tumorigenesis via DNA-PK and Akt. *Nat Med,* 21**,** 906-13.

WIRTZ, S., BECKER, C., BLUMBERG, R., GALLE, P. R. & NEURATH, M. F. 2002. Treatment of T cell-dependent experimental colitis in SCID mice by local administration of an adenovirus expressing IL-18 antisense mRNA. *J Immunol,* 168**,** 411-20.

WIRTZ, S. & NEURATH, M. F. 2007. Mouse models of inflammatory bowel disease. *Adv Drug Deliv Rev,* 59**,** 1073-83.

WONG, J. L., BERK, E., EDWARDS, R. P. & KALINSKI, P. 2013. IL-18-primed helper NK cells collaborate with dendritic cells to promote recruitment of effector CD8+ T cells to the tumor microenvironment. *Cancer Res,* 73**,** 4653-62.

WU, D. & PAN, W. 2010. GSK3: a multifaceted kinase in Wnt signaling. *Trends in Biochemical Sciences,* 35**,** 161-168.

WU, X. F., OUYANG, Z. J., FENG, L. L., CHEN, G., GUO, W. J., SHEN, Y., WU, X. D., SUN, Y. & XU, Q. 2014. Suppression of NF-kappaB signaling and NLRP3 inflammasome activation in macrophages is responsible for the amelioration of experimental murine colitis by the natural compound fraxinellone. *Toxicol Appl Pharmacol,* 281**,** 146-56.

XAVIER, R. J. & PODOLSKY, D. K. 2007. Unravelling the pathogenesis of inflammatory bowel disease. *Nature,* 448**,** 427-34.

XU, X.-R., LIU, C.-Q., FENG, B.-S. & LIU, Z.-J. 2014. Dysregulation of mucosal immune response in pathogenesis of inflammatory bowel disease. *World journal of gastroenterology,* 20**,** 3255-3264.

YAMAMOTO, H., MONDEN, T., MIYOSHI, H., IZAWA, H., IKEDA, K., TSUJIE, M., OHNISHI, T., SEKIMOTO, M., TOMITA, N. & MONDEN, M. 1998. Cdk2/cdc2 expression in colon

carcinogenesis and effects of cdk2/cdc2 inhibitor in colon cancer cells. *Int J Oncol,* 13**,** 233-9.

YAN, Y., JIANG, W., SPINETTI, T., TARDIVEL, A., CASTILLO, R., BOURQUIN, C., GUARDA, G., TIAN, Z., TSCHOPP, J. & ZHOU, R. 2013. Omega-3 fatty acids prevent inflammation and metabolic disorder through inhibition of NLRP3 inflammasome activation. *Immunity,* 38**,** 1154-63.

YAN, Y., KOLACHALA, V., DALMASSO, G., NGUYEN, H., LAROUI, H., SITARAMAN, S. V. & MERLIN, D. 2009. Temporal and spatial analysis of clinical and molecular parameters in dextran sodium sulfate induced colitis. *PLoS One,* 4**,** e6073.

YANG, K., POPOVA, N. V., YANG, W. C., LOZONSCHI, I., TADESSE, S., KENT, S., BANCROFT, L., MATISE, I., CORMIER, R. T., SCHERER, S. J., EDELMANN, W., LIPKIN, M., AUGENLICHT, L. & VELCICH, A. 2008. Interaction of Muc2 and Apc on Wnt signaling and in intestinal tumorigenesis: potential role of chronic inflammation. *Cancer Res,* 68**,** 7313-22.

YAO, X., XING, Y., ZHANG, C., ZHAO, L. & MENG, G. 2016. Hyper inflammasome signaling in Nlrp3 mutant mice alters gut microbiota and confers resistance to colitis and colorectal cancer. *European Journal of Immunology,* 46**,** 278-278.

YAO, X., ZHANG, C., XING, Y., XUE, G., ZHANG, Q., PAN, F., WU, G., HU, Y., GUO, Q., LU, A., ZHANG, X., ZHOU, R., TIAN, Z., ZENG, B., WEI, H., STROBER, W., ZHAO, L. & MENG, G. 2017a. Remodelling of the gut microbiota by hyperactive NLRP3 induces regulatory T cells to maintain homeostasis. *Nature Communications,* 8**,** 1896.

YAO, X. M., ZHANG, C. H., XING, Y., XUE, G., ZHANG, Q. P., PAN, F. W., WU, G. J., HU, Y. X., GUO, Q. H., LU, A. L., ZHANG, X. M., ZHOU, R. B., TIAN, Z. G., ZENG, B. H., WEI, H., STROBER, W., ZHAO, L. P. & MENG, G. X. 2017b. Remodelling of the gut microbiota by hyperactive NLRP3 induces regulatory T cells to maintain homeostasis. *Nature Communications,* 8**,** 17.

YOUM, Y. H., GRANT, R. W., MCCABE, L. R., ALBARADO, D. C., NGUYEN, K. Y., RAVUSSIN, A., PISTELL, P., NEWMAN, S., CARTER, R., LAQUE, A., MUNZBERG, H., ROSEN, C. J., INGRAM, D. K., SALBAUM, J. M. & DIXIT, V. D. 2013. Canonical Nlrp3 inflammasome links systemic low-grade inflammation to functional decline in aging. *Cell Metab,* 18**,** 519-32.

YOUM, Y. H., NGUYEN, K. Y., GRANT, R. W., GOLDBERG, E. L., BODOGAI, M., KIM, D., D'AGOSTINO, D., PLANAVSKY, N., LUPFER, C., KANNEGANTI, T. D., KANG, S., HORVATH, T. L., FAHMY, T. M., CRAWFORD, P. A., BIRAGYN, A., ALNEMRI, E. & DIXIT, V. D. 2015. The ketone metabolite beta-hydroxybutyrate blocks NLRP3 inflammasome-mediated inflammatory disease. *Nat Med,* 21**,** 263-9.

YU, H., PARDOLL, D. & JOVE, R. 2009. STATs in cancer inflammation and immunity: a leading role for STAT3. *Nat Rev Cancer,* 9**,** 798-809.

YU, M., JIA, H. M., ZHOU, C., YANG, Y., SUN, L. L. & ZOU, Z. M. 2017. Urinary and Fecal Metabonomics Study of the Protective Effect of Chaihu-Shu-Gan-San on Antibiotic-Induced Gut Microbiota Dysbiosis in Rats. *Sci Rep,* 7**,** 46551.

ZAKI, M. H., BOYD, K. L., VOGEL, P., KASTAN, M. B., LAMKANFI, M. & KANNEGANTI, T. D. 2010a. The NLRP3 inflammasome protects against loss of epithelial integrity and mortality during experimental colitis. *Immunity,* 32**,** 379-91.

ZAKI, M. H., LAMKANFI, M. & KANNEGANTI, T. D. 2011a. Inflammasomes and Intestinal Tumorigenesis. *Drug Discov Today Dis Mech,* 8**,** e71-e78.

ZAKI, M. H., LAMKANFI, M. & KANNEGANTI, T. D. 2011b. The Nlrp3 inflammasome: contributions to intestinal homeostasis. *Trends Immunol,* 32**,** 171-9.

ZAKI, M. H., VOGEL, P., BODY-MALAPEL, M., LAMKANFI, M. & KANNEGANTI, T. D. 2010b. IL-18 Production Downstream of the Nlrp3 Inflammasome Confers Protection against Colorectal Tumor Formation. *Journal of Immunology,* 185**,** 4912-4920.

ZAKI, M. H., VOGEL, P., MALIREDDI, R. K., BODY-MALAPEL, M., ANAND, P. K., BERTIN, J., GREEN, D. R., LAMKANFI, M. & KANNEGANTI, T. D. 2011c. The NOD-like receptor NLRP12 attenuates colon inflammation and tumorigenesis. *Cancer Cell,* 20**,** 649-60.

ZAMBETTI, L. P. & MORTELLARO, A. 2014. NLRPs, microbiota, and gut homeostasis: unravelling the connection. *Journal of Pathology,* 233**,** 321-330.

ZHANG, J., FU, S., SUN, S., LI, Z. & GUO, B. 2014a. Inflammasome activation has an important role in the development of spontaneous colitis. *Mucosal Immunology,* 7**,** 1139-1150.

ZHANG, L., MO, J., SWANSON, K. V., WEN, H., PETRUCELLI, A., GREGORY, S. M., ZHANG, Z., SCHNEIDER, M., JIANG, Y., FITZGERALD, K. A., OUYANG, S., LIU, Z. J., DAMANIA, B., SHU, H. B., DUNCAN, J. A. & TING, J. P. 2014b. NLRC3, a member of the NLR family of proteins, is a negative regulator of innate immune signaling induced by the DNA sensor STING. *Immunity,* 40**,** 329-41.

ZHAO, S., GONG, Z., ZHOU, J., TIAN, C., GAO, Y., XU, C., CHEN, Y., CAI, W. & WU, J. 2016. Deoxycholic Acid Triggers NLRP3 Inflammasome Activation and Aggravates DSS-Induced Colitis in Mice. *Front Immunol,* 7**,** 536.

ZHAO, Y., GUO, Q., ZHAO, K., ZHOU, Y., LI, W., PAN, C., QIANG, L., LI, Z. & LU, N. 2018. Small molecule GL-V9 protects against colitis-associated colorectal cancer by limiting NLRP3 inflammasome through autophagy. *OncoImmunology,* 7**,** e1375640.

ZHAO, Y. & SHAO, F. 2015. The NAIP-NLRC4 inflammasome in innate immune detection of bacterial flagellin and type III secretion apparatus. *Immunol Rev,* 265**,** 85-102.

ZHERNAKOVA, A., FESTEN, E. M., FRANKE, L., TRYNKA, G., VAN DIEMEN, C. C., MONSUUR, A. J., BEVOVA, M., NIJMEIJER, R. M., VAN 'T SLOT, R., HEIJMANS, R., BOEZEN, H. M., VAN HEEL, D. A., VAN BODEGRAVEN, A. A., STOKKERS, P. C., WIJMENGA, C., CRUSIUS, J. B. & WEERSMA, R. K. 2008. Genetic analysis of innate immunity in Crohn's disease and ulcerative colitis identifies two susceptibility loci harboring CARD9 and IL18RAP. *Am J Hum Genet,* 82**,** 1202-10.

ZHOU, R., YAZDI, A. S., MENU, P. & TSCHOPP, J. 2011. A role for mitochondria in NLRP3 inflammasome activation. *Nature,* 469**,** 221-5.

ZHU, S., DING, S., WANG, P., WEI, Z., PAN, W., PALM, N. W., YANG, Y., YU, H., LI, H. B., WANG, G., LEI, X., DE ZOETE, M. R., ZHAO, J., ZHENG, Y., CHEN, H., ZHAO, Y., JURADO, K. A., FENG, N., SHAN, L., KLUGER, Y., LU, J., ABRAHAM, C., FIKRIG, E., GREENBERG, H. B. & FLAVELL, R. A. 2017. Nlrp9b inflammasome restricts rotavirus infection in intestinal epithelial cells. *Nature,* 546**,** 667-670.

ZIEGLER, S. F. & BUCKNER, J. H. 2009. FOXP3 and the regulation of Treg/Th17 differentiation. *Microbes Infect,* 11**,** 594-8.

APPENDICES

Appendix-1: Copy of Ethics Approval Permits

UNIVERSITY of TASMANIA

Animal Ethics Committee
ETHICS APPROVAL PERMIT

Office of Research Services
Phone : 03 62267283
Fax: 03 62267148
animal.ethics@utas.edu.au

To: Dr Raj Eri

From: Natasha Jones

Date: 20 March 2017

Project: A0016166 - Understanding the progression of colitis to colorectal cancer

Approved on: 06 March 2017

Approval expires: 05 March 2020

1st Annual Report due: 06 March 2018

Please read this permit carefully as **approval may be withdrawn** for non-compliance with the conditions stated below.

The Animal Ethics Committee has approved the above project and a copy of the initial application document is attached. The approval is subject to the review and AEC approval of an annual report which is due before the approval anniversary. **Please note the due date in your diary.**

As the Responsible Investigator, you MUST ensure that:

1. All aspects of the work conform to the requirements of the current edition of the *Australian code of practice for the care and use of animals for scientific purposes* 8th edition 2013

2. The project is conducted in accordance with the provisions of the Tasmanian Veterinary Surgeons Act 1987 and Veterinary Surgeons Regulations 2012. If the project involves a veterinary service or other animal service, it is **your responsibility** to contact the University Veterinarian to discuss the legal requirements of competency assessment.

3. The University Veterinarian and the Animal Ethics Committee are promptly notified of any unexpected event which was not considered in

UTAS	University of Tasmania Animal Ethics Committee **ETHICS APPROVAL PERMIT**	University of Tasmania Office of Research Services Ph: 03 62267283 Fax: 03 62267148 animal.ethics@utas.edu.au

To:	Dr Raj Eri
From:	Marilyn Pugsley Executive Officer Animal Ethics
Date:	30 June 2014
Project:	A14095 – Expression of Inflammasome Components in NLRP3 and NLRP6 Knockout mice
Approved on:	30 June 2014
Approval expires:	30 June 2017
1st Annual Report due before:	30 June 2015

Please read this permit carefully as **approval may be withdrawn** for projects that do not comply with the conditions

NOTE SPECIAL CONDITION DETAILED BELOW

The RI must inform the AEC when the PC2 status is granted and when an IBC permit has been obtained. Transgenic mice must not be taken to Launceston before this occurs.

The Animal Ethics Committee has approved the above project and a copy of the document is attached. The approval is subject to the review and approval of an annual report which is due before the approval anniversary. **Please note this date in your diary.**

This approval constitutes ethical clearance by the Animal Ethics Committee. If this project involves the conduct of a Veterinary Service or Other Animal Service as defined in the Veterinary Surgeons Act 1987 (Tas) and Veterinary Surgeons Regulations 2012 (Tas), it is your responsibility to ensure that the project is conducted in accordance with the provisions of the Act and Regulations. Please contact the Animal Welfare Officer, Dr Sue Ottomanski (6226 7491 or sue.ottomanski@utas.edu.au) to discuss veterinary procedure competency accreditation.

As the Responsible Investigator, you MUST ensure that:

(a) all aspects of the work conform to the requirements of the current edition of the *Australian code of practice for the care and use of animals for scientific purposes* 8th edition 2013

(b) a full record is maintained of all animals used in this project. If at any stage you anticipate the need to use additional animals this must be communicated to the

Appendix-2: Copies of conferences posters

Major role of NLRP3 inflammasome in colitis

UNIVERSITY of TASMANIA AUSTRALIA

Agampodi Promoda Perera, Nicole Ranson, Dr.Raj Eri, Sukhwinder S Sohal

Introduction

Colitis

- Inflammation of the colon
- Symptoms- diarrhoea
  loss of body weight
  abdominal cramps
- Negative shift in the microbial community

Spontaneous Colitis Mouse Model

Winnie

- missense mutations in Muc2 gene
- aberrant Muc2 leading to ER stress
- diminished mucus barrier
- spontaneous intestinal inflammation

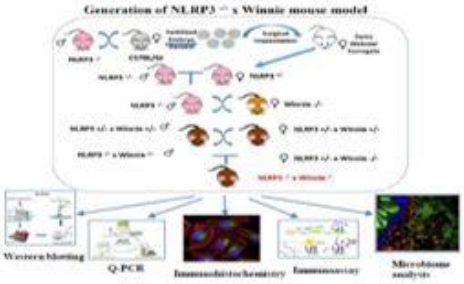
The role of NLRP3 in chemically induced colitis in mice is contradictory.

Hypothesis: Lack of NLRP3 in spontaneous colitis mouse model *winnie* will ameliorate colitis

- Aim 1: Generation and phototyping of NLRP3$^{-/-}$ x *Winnie*
- Aim 2: Influence of NLRP3 on gut microbiota
- Aim 3: Efficacy and action of small molecule NLRP3 inhibitors

Methods

Generation of NLRP3$^{-/-}$ x Winnie mouse model



Inflammasome activation assay

Results

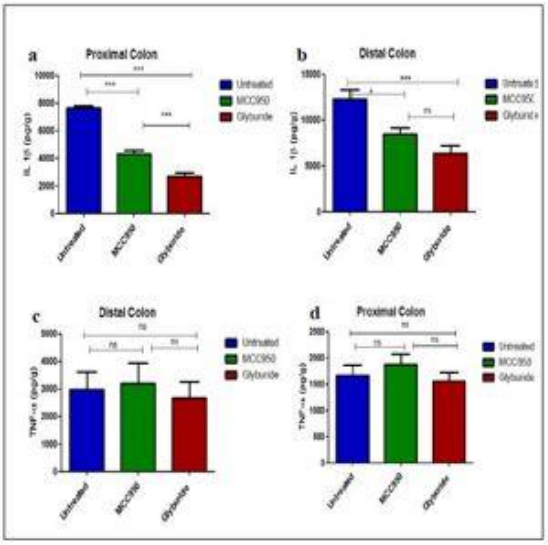


Figure 1: Production of IL-1β (a,b) and TNF-α (c,d) from *Winnie* colon explants stimulated with LPS treated with MCC950 (0.1uM) and Glyburide (200uM) measured by ELISA. Data are expressed as the mean ± sem of 5 independent experiments carried out in duplicate.

Discussion

We found a significant reduction in IL-1 β expression with MCC950 and Glyburide treatment suggesting that they effectively inhibits the activation of NLRP3 inflammosome in a chronic colitis model. TNF-α expression was not affected by the drugs ruling out a general defect in macrophage responsiveness.

Research Outcome

The novel spontaneous colitis model NLRP3$^{-/-}$ x *Winnie* will give a comprehensive understanding of the role of NLRP3 inflammasome in the disease pathogenesis of colitis. For the first time it is shown that MCC950 and Glyburide effectively blocks NLRP3 inflammasome activation in a chronic colitis model *winnie*. This has potential therapeutic implication not only in inflammatory bowel disease but also in colitis associated colorectal cancer.

References

- Heazlewood, C.K., et al., Aberrant mucin assembly in mice causes endoplasmic reticulum stress and spontaneous inflammation resembling ulcerative colitis. PLoS Med, 2008. 5(3): p. e54.
- Coll, R.C., et al., A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases. Nat Med, 2015. 21(3): p. 248-255.
- Hirota, S.A., et al., NLRP3 inflammasome plays a key role in the regulation of intestinal homeostasis. Inflamm Bowel Dis, 2011. 17(6): p. 1359-72.

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Australasian Society for Immunology Inc.

IUIS International Union of Immunological Societies

ICI 2016 International Congress of Immunology 21-26 August 2016 Melbourne Australia

UNIVERSITY of TASMANIA

NLRP3- A key player at the tipping point of colitis into colitis-associated colorectal cancer

Agampodi Promoda Perera [1],Ben Southam [1],Sam Brake [1],Sukhwinder Singh Sohal [1],Ruchira Fernando [2],Dale Kunde[1], Rajaraman Eri[1]
School of Health Sciences, University of Tasmania[1], Pathology, Launceston General Hospital[2]

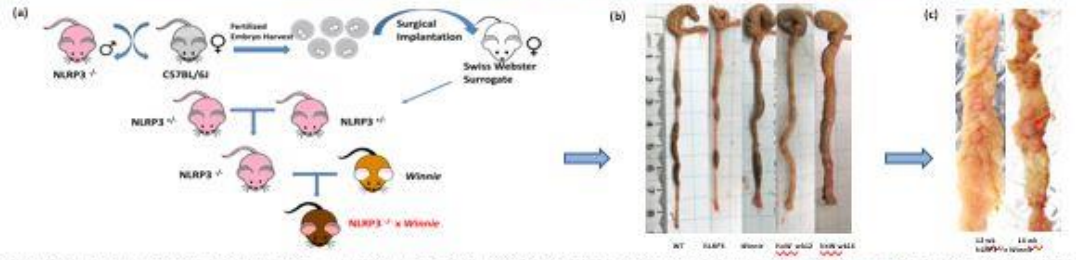


Figure 1. (a) Schematic diagram of NLRP3 -/- x *Winnie* generation. (b) Representative photographs of colons from Wild Type,NLRP3 -/- ,*Winnie* , NLRP3 -/- x *Winnie* (c) Representative images of colon tumors at 12 week and 16 week NLRP3 -/- x *Winnie*.

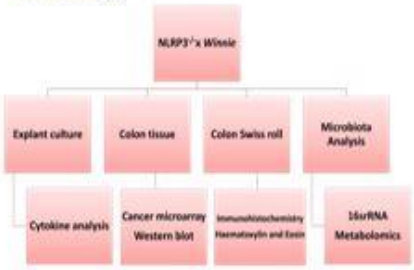
Introduction

Ulcerative colitis is associated with colorectal cancer but the exact mechanism of how chronic inflammation initiates colorectal cancer has not been established. Studies have indicated a role for NLRP3 inflammasome in colitis and tumorigenesis but the results have been controversial due to different methods of chemical induction. To address the controversial role of NLRP3 we have designed a murine model deficient in NLRP3 in a spontaneous chronic colitis mouse model *Winnie*[1] (*Muc2* mutant). This project will be the first study to model NLRP3 regulated molecular changes in inflammation-associated colorectal cancer without the need for carcinogen treatment.

Objectives

- Generate the spontaneous tumorigenesis model NLRP3 $^{-/-}$x *Winnie*.
- Detect immunological, molecular and microbiota changes to identify specific mechanisms involved in NLRP3 $^{-/-}$x *Winnie* tumorigenesis.
- Block NLRP3 using specific small molecule NLRP3 inhibitor MCC950, in *Winnie* mice to define the role of NLRP3 in colitis.

Methodology



Summary

- In the absence of NLRP3 chronic inflammation progress to colorectal cancer.
- NLRP3 $^{-/-}$x *Winnie* colon tissue display adenoma and invasive carcinoma arising from severely dysplastic lesions.
- NLRP3 $^{-/-}$x *Winnie* shows high levels of oxidative stress and DNA damage consistent with colorectal cancer.
- NLRP3 $^{-/-}$x *Winnie* shows increased levels of IL-1β indicating NLRP3 independent infalmmasome activation.

Results

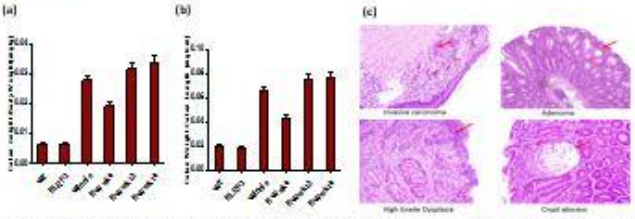


Figure 2. (a) Colon length to Body Weight ratio (b) Colon Weight to Colon Length ratio. Data presented as mean± s.e.m (n=12 mice/group (c) Haematoxylin and Eosin stain of colon sections of 12 week NLRP3 $^{-/-}$ x *Winnie*

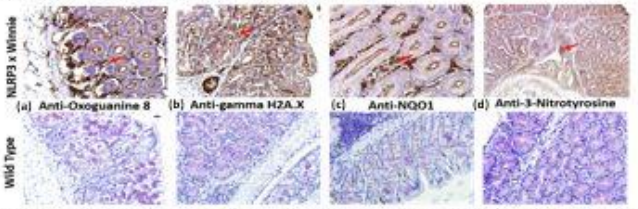


Figure 3. Immunohistochemical staining of DNA damage markers (a, b) and Oxidative stress markers (c, d) Arrows indicate strong staining of DNA lesions in cells between crypts.

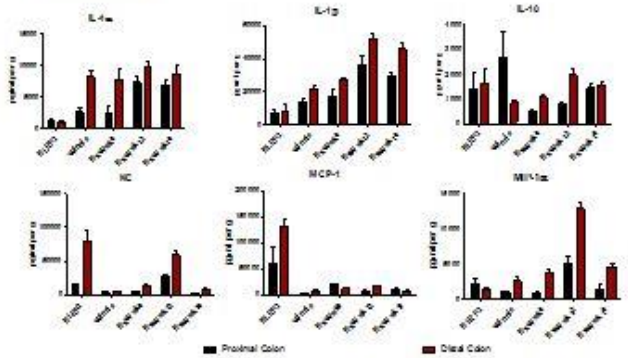


Figure 4. Levels of cytokines in distal and proximal colon explant tissue culture media (n=3). Data presented as mean± s.e.m

Future Directions

- Identify the tumour suppressive molecular pathway of NLRP3
- Analyse the effect of NLRP3 on gut microbiota and homeostasis
- Identify the relative effects of IL-1β and IL-18 in Colorectal cancer
- Efficacy of anti-colitis therapies in cancer progression

References

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NLRP3 a double edged sword in colitis-associated cancer

Agampodi Promoda Perera [1], Avril A.B Robertson [2], Rajaraman Eri[1]
School of Health Sciences, University of Tasmania[1], Institute for Molecular Bioscience, University of Queensland[2].

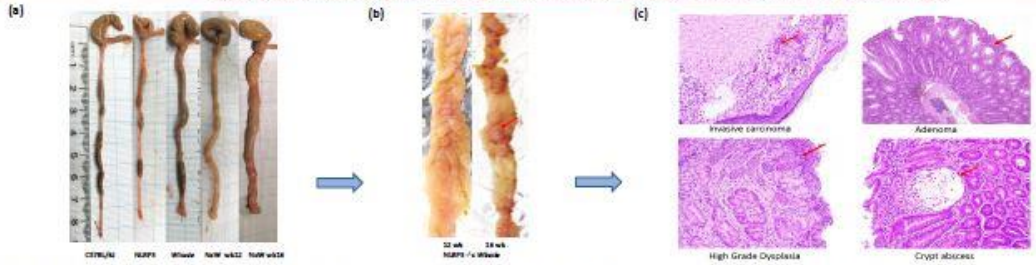

Figure 1. (a) Representative photographs of colons from C57BL/6J, NLRP3$^{-/-}$, *Winnie*, NLRP3$^{-/-}$ x *Winnie* (b) Representative images of colon tumors at 12 week and 16 week NLRP3$^{-/-}$ x *Winnie* (c) Haematoxylin and Eosin stain of colon of 12 week NLRP3$^{-/-}$ x *Winnie*

Introduction

Ulcerative colitis is associated with colorectal cancer but the exact mechanism of how chronic inflammation initiates colorectal cancer has not been established. To address the controversial role of NLRP3 we have designed a murine model deficient in NLRP3 in a spontaneous chronic colitis mouse model *Winnie*[1] (*Muc2* mutant). This project will be the first study to model NLRP3 regulated molecular changes in inflammation-associated colorectal cancer without the need for carcinogen treatment. We also looked at chemical inhibition of NLRP3 in the Winnie colon with MCC950, a potent highly specific small molecule inhibitor.

Objectives

- Generate the spontaneous tumorigenesis model NLRP3 $^{-/-}$ x *Winnie*.
- Detect immunological, molecular and microbiota changes to identify specific mechanisms involved in NLRP3 $^{-/-}$ x *Winnie* tumorigenesis.
- Block NLRP3 using specific small molecule NLRP3 inhibitor MCC950, in *Winnie* mice to define the role of NLRP3 in colitis.

Results

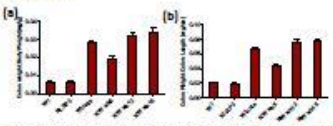

Figure 2. (a) Colon length to Body Weight ratio (b) Colon Weight to Colon Length ratio.

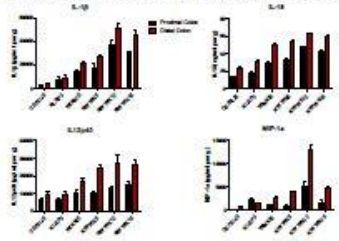

Figure 3. Levels of cytokines in distal and proximal colon explant tissue culture media.

Summary

- In the absence of NLRP3 chronic inflammation progress to colorectal cancer.
- Oral administration of MCC950 to an over active NLRP3 inflammasome in chronic colitis attenuated severity of the disease.

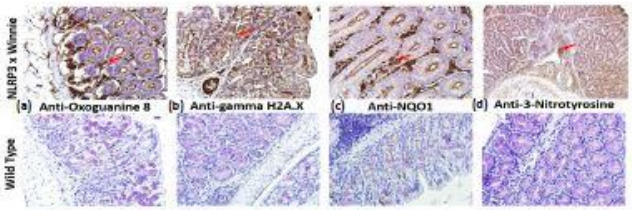

Figure 4. Immunohistochemical staining of DNA damage markers (a, b) and Oxidative stress markers (c, d) Arrows indicate strong staining of DNA lesions in cells between crypts.

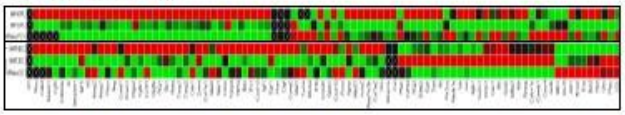

Figure 5. microarray clustergram showing magnitude of expression of colon cancer genes in proximal and distal colon tissue n=3.

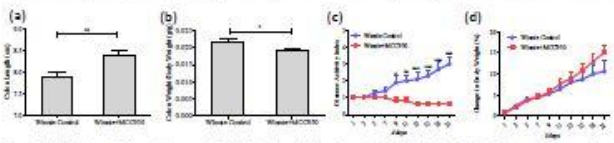

Figure 6. (a) Colon Length for each group. (b) Ratio of colon weight over body weight. (c) Body weight of mice was measured every 3 days and presented as a percentage of their initial weight. (d) Disease activity index.Data are represented as means ± SEM (n=10 per group

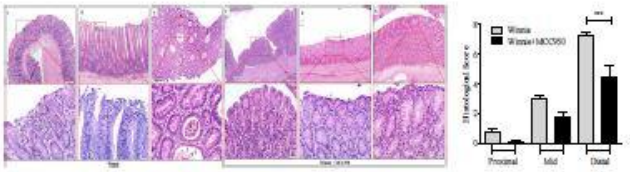

Figure 7. (c-e) Representative Winnie control (f-h) Representative MCC950 treated proximal, middle and distal colon sections stained with hematoxylin and eosin at 100x and 400x.The graph is the summed inflammation scores of control and treated Winnie n=10 per group

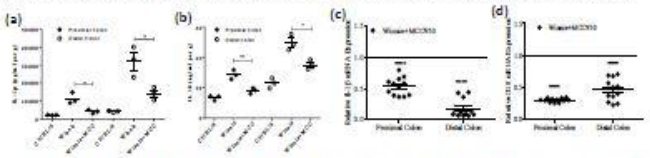

Figure 8. Cytokine levels (a) IL-1β (b) IL18 in proximal and distal colon explant supernatants. The mRNA expressions of (c) IL-1β (d) IL18 in proximal and distal colon tissue. The MCC950 treated data was normalised to the control samples.

Future Directions

- Identify the tumour suppressive molecular pathway of NLRP3
- Analyse the effect of NLRP3 on gut microbiota and homeostasis
- Efficacy of anti-colitis therapies in cancer progression

References

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MCC950 attenuates colonic inflammation in spontaneous colitis mice

Agampodi Promoda Perera [1], Avril A.B Robertson [2], Rajaraman Eri[1]
School of Health Sciences, University of Tasmania[1], Institute for Molecular Bioscience, University of Queensland[2].

Introduction

MCC950 is a potent, highly specific small molecule inhibitor of canonical and noncanonical activation of NLRP3 inflammasome. In the present study for the first time we investigated the effect of MCC950 in a spontaneous chronic colitis mouse model *Winnie*, which mimics human ulcerative colitis.

Aim

Investigate the efficacy and mechanism of specific small molecule NLRP3 inhibitor MCC950 in *Winnie* mice to define the role of NLRP3 in colitis.

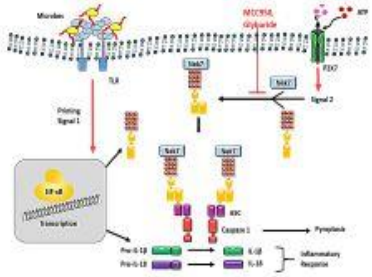

Figure 1: Schematic illustration of the activation of NLRP3 inflammasome.

Results

The effect of MCC950 on NLRP3 inflammasome activation in murine macrophages

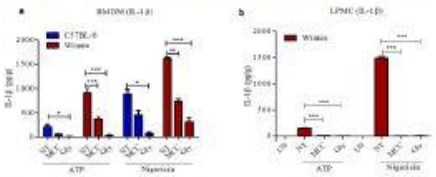

Figure 2: Production of IL-1β (a) C57BL/6 and Winnie BMDMs 0.01 μM MCC95 (b) Winnie LPMCs with MCC950 1 μM as measured by ELISA. Data are expressed as the mean ± sem of three independent experiments carried out in duplicates.

MCC950 inhibits NLRP3 inflammasome activation in colonic explants

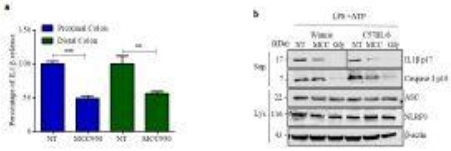

Figure 3: (a) Percentage of IL-1β release of Winnie proximal and distal colons with no treatment compared to treated with MCC950 (10 μM) as measured by ELISA. Data are expressed as the mean ± SEM of five independent experiments carried out in duplicates. (b) Western blots of tissue lysates and supernatants from proximal and distal colons treated with MCC950 (1 μM) or glyburide (200 μM).

Oral administration of MCC950 attenuates colonic inflammation in Winnie

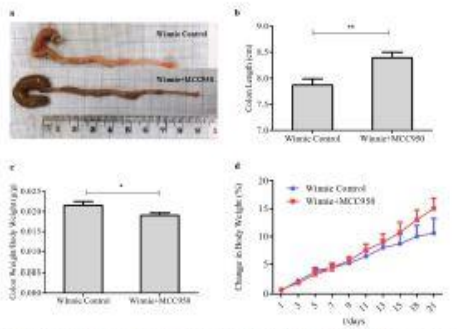

Figure 4: (a) Macroscopic appearances and (b) Colon Length for each group. (c) Ratio of colon weight over body weight. Data are expressed as the mean ± SEM (d) Body weight of mice presented as a percentage of their initial weight. Data are represented as means ± SEM (n=10 per group).

MCC950 treatment improves colitis in 10 week old Winnie

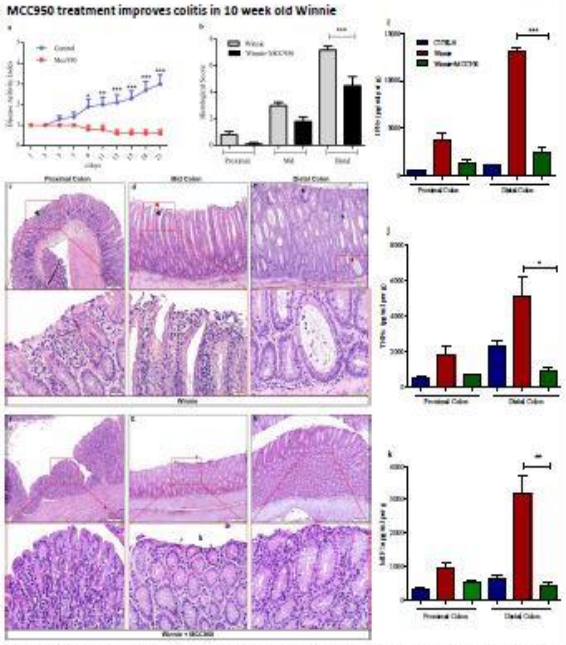

Figure 5. a) Disease activity index. (b) Comparison of summed inflammation scores between control and treatment Winnie mice. (c) Lamina propria inflammatory cell infiltrates (black arrow) (d) epithelial surface damage (red arrow), goblet cell loss (black arrow) (e) Crypt abscesses (red arrow) or damaged epithelium and complete crypt loss (blue arrow) and crypt architectural distortion (black arrow). (f-h) Representative MCC950 treated Winnie colon sections stained with hematoxylin and eosin at 100x and 400x. Protein levels of cytokines (i) IFNγ (j) TNF-α and (k) MIP1a in explant supernatants were determined by Bio-plex. Data are presented as means ± SEM (n=3)

MCC950 suppressed NLRP3 activated proinflammatory cytokine levels in colon explant of Winnie

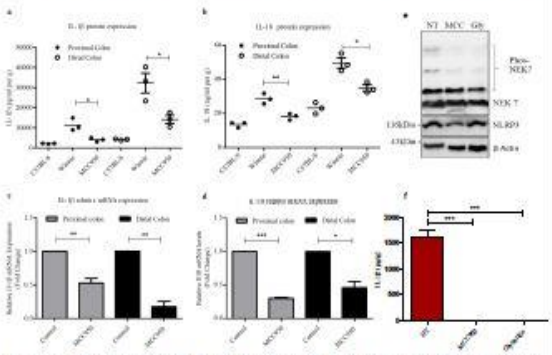

Figure 6. Protein levels of cytokines (a) IL-1β (b) IL18 in proximal and distal colon explant supernatants as determined by Bio-plex. The mean values of fold change in mRNA expression levels for (c) IL-1β (d) IL18 in MCC950 treated Winnie proximal and distal colon tissue. (e)The phosphorylation state of NEK7 was analysed using Phos-tag SDS-PAGE. These results are representative of three independent experiments. (f) ELISA analysis of IL-1β in the culture supernatant of J774A.1 cells treated as in (e).

Summary

Oral administration of MCC950 to an over active NLRP3 inflammasome in chronic colitis attenuated severity of the disease.

Future Directions

- Effect of MCC950 on Human colon explants
- Efficacy of anti-colitis therapies in cancer progression

Acknowledgement

Australian Postgraduate Award from University of Tasmania for Agampodi Promoda Perera for postgraduate research is acknowledged.

Reference

Perera, A.P., et al., MCC950, a specific small molecule inhibitor of NLRP3 inflammasome attenuates colonic inflammation in spontaneous colitis mice. Scientific Reports, 2018. 8(1): p. 8618.
[2] Zaki, M.H., et al., IL-1 receptor antagonist-deficient mice are resistant to DSS-induced colitis and cancer development. Gut Biol, 2011. 1(1): p. 1-9.

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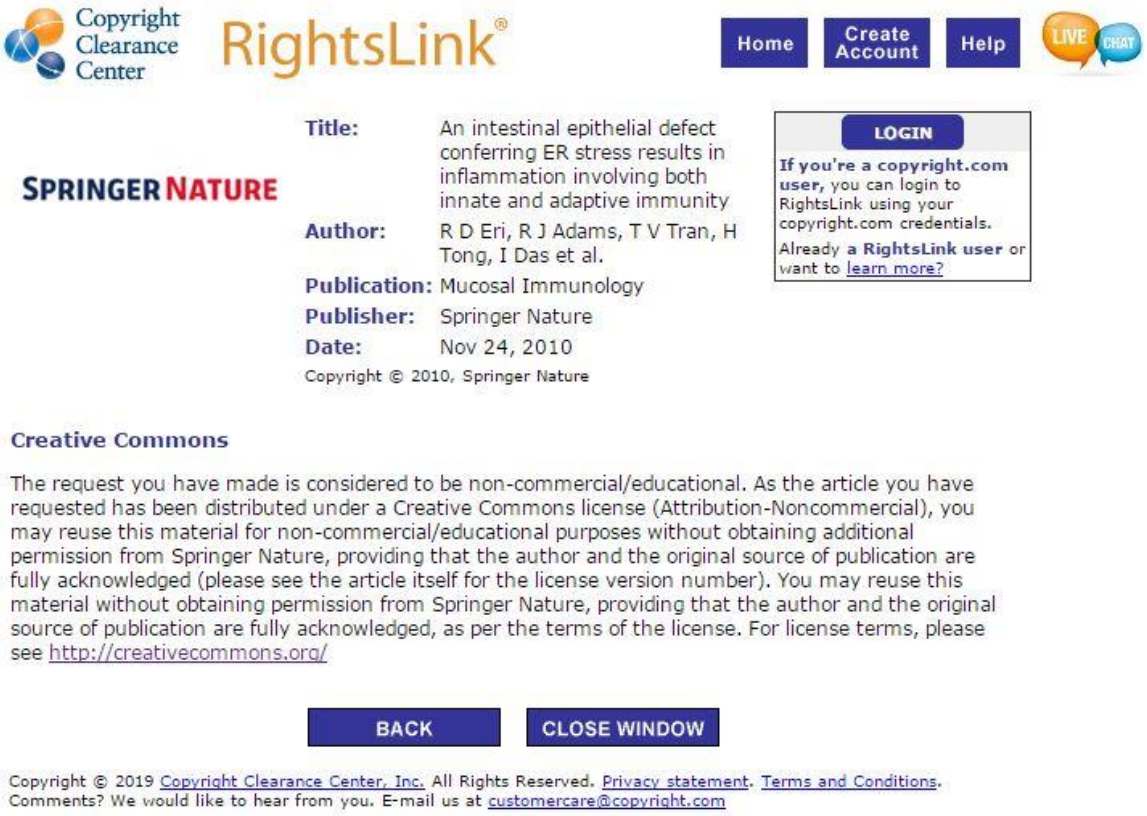

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Title: An intestinal epithelial defect conferring ER stress results in inflammation involving both innate and adaptive immunity

Author: R D Eri, R J Adams, T V Tran, H Tong, I Das et al.

Publication: Mucosal Immunology

Publisher: Springer Nature

Date: Nov 24, 2010

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